Process development of enzyme catalysed industrial production of partial acylglycerols

Damstrup, Marianne

Publication date:
2008

Document Version
Publisher's PDF, also known as Version of record

Process Development of Enzymatic Glycerolysis for Industrial Monoacylglycerol Production

Marianne Linde Damstrup
PhD Thesis

2008

National Food Institute
Food Production Engineering
Technical University of Denmark

Preface

This thesis is part of the requirements for obtaining the Ph.D. degree at the Technical University of Denmark (DTU). The Ph.D. project behind this thesis (entitled 'Development of an Enzyme-catalyzed Industrial Applicable Process for Partial Acylglycerol Production') was carried out from November 2003 to July 2008. The 3 years full-time study was prolonged by a requested part-time employment from November 2004 and one year of maternity leave from November 2006 to November 2007. The Ph.D. project was based on a research agreement assigned in 2003 between the sponsor of the project Danisco A/S, Brabrand, Denmark and BioCentrum-DTU, Lyngby, Denmark (Now DTU Biosys).

The Ph.D. study was mainly performed at BioCentrum-DTU, under main supervision from Xuebing Xu, associate Professor at BioCentrum-DTU. From January 2008, a merger between Department of Food Production Engineering, DTU Biosys and other Food Research Groups outside DTU resulted in a new group affiliation to Food Production Engineering, National Food Institute, Lyngby, Denmark. Xuebing Xu started in a new position as Professor in Agro-Biotechnology Science, Department of Molecular Biology, University of Aarhus from February 2008. Hence, from here Jens Adler-Nissen, Professor, National Food Institute took over the role as main supervisor while Xuebing Xu, was assigned as co-supervisor. The project was performed in collaboration with Department of Chemical and Biochemical Engineering at DTU under supervision from Professor Anker Degn Jensen and associate Professor Søren Kiil. The analytical GC-FID analyses and the pilot plant experiments were conducted at Danisco A/S' facilities, Brabrand, Denmark under supervision from Flemming Vang Sparsø, Senior Scientist, Emulsifier Division. This industrial collaboration resulted in regularly visits at Danisco A/S, Brabrand.

The project has involved a number of people at BioCentrum-DTU (Now DTU Biosys), the National Food Institute, Department of Chemical and Biochemical Engineering and Danisco A/S. To all, I would like to give my gratitude for their great support and kind help during the study. In particular, I would like to acknowledge my five supervisors Xuebing Xu, Jens Adler-Nissen, Flemming Vang Sparsø, Anker Degn Jensen and Søren Kiil for general guidance, useful advice and fruitful

discussions. A special thanks to Jens Adler-Nissen for his engagement as main-supervisor in the last months although he entered the project at a very late stage. Xuebing Xu is gratefully thanked for supporting me until the very end of the project even after he started his new work at University of Aarhus. Furthermore, Xuebing Xu is thanked for encouraging me to apply for the achieved American Oil Chemists' Society (AOCS) Biotechnology student excellence award 2008. Jens Abildskov is greatly acknowledged for his valuable support when using thermodynamic models. A special thanks to Anette Gravgaard for financing the project and allowing me to use Danisco A/S's facilities. A specific acknowledgement to Bodil Alrø for her excellent help with the GC-FID analyzes and to Pouel Andersen for his valuable technical assistance with pilot plant experiments. Anni Jensen is thanked for technical assistance with the analytical work and for great support during the first year of the study. Tine Jensen and other involved students are thanked for their interest in this particular field. Lars Damstrup is greatefully acknowledged for the time spent on revising the thesis. Nancy Kjøbæk is thanked for designing the front page.

Last, I would like to express my gratitude to my husband Mikkel and our three beloved children, Sebastian, Christina and Benjamin. Without their great support, love and understanding I could not have succeeded this study.

July 2008

Lyngby, Denmark

Marianne Linde Damstrup

Table of contents

Abstract

In the present Ph.D. project, an enzyme catalyzed 'bioprocess' for monoacylglycerol (MAG) production, feasible for implementation in industrial facilities, was developed. It was a simple operated and efficient lipase catalyzed glycerolysis reaction between oil carrying nutritional important polyunsaturated fatty acids (PUFA) and glycerol in an enzyme packed bed reactor. Subsequently, the formed MAGs were purified. Different aspects of the developed process were investigated to obtain an in-depth understanding of the system.

The comercially availible immobilized *Candida antarctica* lipase B (Novozym®435) demonstrated excellent catalytic properties in the developed process. A filling amount of approximately 220 kg per m^3 column was appropiate for long lasting continuous reaction which took the enzyme swelling during intial reactant wetting into account. Novozym®435 exhibited a capacity of 2 tons MAG/kg enzyme used and a long life time estimated at 2200 h. This excellent lipase perfomance compensated for the high enzyme costs and made its use reasonable from economical point of view. The enzyme demonstrated non-specific catalytic properties and the sn-ratio of the formed MAGs ended with a sn-1 (3):sn-2 ratio of the MAGS of approximately 90:10.

In the reaction mixture solvent addition was needed to ensure a sufficient dispersion of glycerol into oil. A mixture of *tert*-butanol and *tert*-pentanol mixture (80:20 vol%) was the organic media found most beneficial with respect to polarity, physical properties and prices compared to the reaction performance and practical feasibility. The presence of 50 wt% solvent was required to reach equilibrium conditions in desirable short time of just 20 min at 40°C.

A glycerol to oil molar ratio of 4-5 was found optimal to obtain maximal MAG content of 55 wt%, a high conversion degree of the TAG from the oil (97 wt%) and relatively low DAG and glycerol contents in the product mixture (of 16 and 20 wt%, respectively). A few weight percentages fatty acids and fatty acid esters were formed during reaction. This was ascribed small impurities amounts from the components included in the system (secondary alcohols, water and saturated FA from the organic media, glycerol, enzyme and oil). From kinetical studies the conversion of TAG to DAG seemed to be the rate limiting step of the glycerolysis reaction. No distinct internal mass transfer problems with restricted reactant access to the active lipase site occurred when the immobilzed enzyme was supplied in the particle range obtained from the manufacturer. Column length to diamter ratio < 10 and fluid velocities in range from $1.7 \cdot 10^{-5}$ to $5.0 \cdot 10^{-5}$ m/s did not result in external mass transfer limitations with poor transfer of the reactant- and product-mixture through the column.

It deemed possible to remove glycerol and solvent by steam stripping under vacuum and subsequent to re-use them. In contrast, trials with glycerol and solvent removal by the use of ultfrafiltration was unsuccesful. Short path distillation (SPD) was found suited to separate the MAG containing PUFAs from the other lipids components to obtain MAGs in a highly pure form (≥ 95%).

The developed process was implemented into a pilot plant facility. High pressure drops were observed when long thin column reactors were used containg 'long' enzyme beds that should be passed. In contrast, a shallow and wide enzyme bed caused no problems with pressure drops which made this set up most well suited.

Resumé

I det gennemførte Ph.D. projekt blev der udviklet en industriel enzym katalyseret 'bio-proces' til kontinuert fremstilling af monoglycerider (MAG). Processen indeholdt en lipase katalyseret glycerolyse reaktion mellem glycerol og vegetabilsk olie, der er rig på triglycerider med en umættet fedtsyre (FA) profil. Reaktionen blev udført i en enzym pakket kolonne reaktor. Herefter fulgte oprensning af det dannede MAG. Forskellige aspekter af processen blev undersøgt med henblik på at opnå en dybtegående forståelse af reaktionssystemet.

Den kommercielt tilgængelige immobiliserede *Candida antarctica* lipase B (Novozym®435) blev anvendt som biokatalysator under glycerolyse reaktionen. En kolonne fyldning på 220 kg enzyme per m^3 kolonne var fundet passende til en længerevarende kontinuert reaktion, der tog højde for opsvulmning af enzymet under indledende vådgøring med reaktant blanding. Novozym®435 havde en MAG produkt kapacitet på 2.2 tons MAG/kg forbrugt enzym og en estimeret 'levetid' på 2200 timer. Disse egenskaber kompenserede for de meget høje kostpriser på enzymet. Enzymet klippede uspecifikt på de forskellige sn-positionerede fedtsyrer og sn-forholdet af de dannede MAG'er var ca. 90:10 sn-1 (3):sn-2 MAGS.

Det blev fundet nødvendigt at iblande reaktionsblandingen opløsningsmiddel for at opnå tilstrækkelig god blanding af glycerol og olie. En blanding af *tert*-butanol og *tert*-pentanol (80:20 vol%) blev vurderet som bedst egnet på baggrund af polaritet, fysiske egenskaber og pris sammenholdt med reaktions effektivitet og håndterbarhed. Tilstedværesle af 50 wt% opløsningsmiddel var nødvendig for at opnå ligevægtsbetingelser indenfor en tilfredstillende kort reaktionstid på 20 min ved 40°C.

Glycerol blandet med olie i et molforhold på 4-5 resulterede i et maksimalt MAG indhold på 55 wt%, en høj omdannelsesgrad af oliens TAG (97%) og et DAG og glycerol indhold i produkt blandingen (på henholdsvis 16 og 20 wt%). Der bleve dannet nogle få % fedtsyrer og fedtsyreestre under reaktionen, hvilket formodentlig skyldes en reaktion med små mængder urenheder i de ingående komponenter (sekundære alkoholer og vand fra opløsningsmiddel, glycerol, enzym og olien). Studier af reaktionskinetikken viste, at TAG omdannelsen til DAG tilsyneladende var det hastighedsbegrænsende trin i glycerolyse reaktionen. Brug af enzymet i dets kommercielt tilgængelige partikel størrelse fordeling ledte ikke til udtalte 'interne masse overførsels problemer' med hæmmet reaktant adgang til det aktive sted på enzymet. Kolonne længde-diameter forhold < 10 og væskestrømningshastigheder fra $1.7 \cdot 10^{-5}$ til $5.0 \cdot 10^{-5}$ m/s afstedkom ingen nævneværdig 'ekstern masseoverførsels begrænsninger' med en inkonsistent reaktant – og produkt gennemstrømning i kolonnen.

Glycerol og opløsningsmiddel kunne fint fjernes fra produkt blandingen vha. 'damp afstripning' og efterfølgende genanvendes. Fjernelse af glycerol og solvent vha. ultrafiltering mislykkedes derimod. 'Kort vejs distillation' var fundet velegnet til at separere MAG indeholdende PUFAs fra de øvrige acylglyceroler (DAG og TAG), således at en høj MAG renhed kunne opnås (≥ 95%).

Den udviklede proces var implementerbar til pilot anlæg. Der opstod dog store tryktab over kolonnen ved brug af tynde kolonner med 'lang' enzym passage vej. Derfor var en reaktor med en 'kort' 'enzyme bed' fundet mest hensigtsmæssig til at minimere tryktab over kolonnen.

Abbreviations used

AHA	American Health Association
b. p.	Boiling Point
CALB	*Candida antarctica* lipase B
cH	cyclo-Hexane
DAG	Diacylglycerol
DSC	Differential Scanning Calometry
FA	Fatty Acid
FAE	Fatty Acid Ester
FDA	U.S. Food and Drug Administration
FFA	Free Fatty Acid
FID	Flame Ionization Detector
FNB	Food and Nutrition Board, Institute of Medicine of the National Academies.
GC	Gas Chromatography
Gly/gly	Glycerol
IS	Internal Standard
LCFA	Long Chain Fatty Acid
l/d	Length-to-diameter
MAG	Monoacylglycerol
nH	n-Hexane
m. p.	Melting Point
MS	Mass Spectrometry
MWCO	Molecular weight Cut-Off
P	Octanol-Water partition coefficient
PET	Polyethyleneterephthalate
PMMA	Poly Methyl Methacrylate
PP	Poly Propelene
PSf	Polysulphone
PUFA	Poly Unsaturated Fatty Acid
PVDF	Polyvinylidenefluoride
R_f	Response Factor
RSM	Response Surface Methodology
SPD	Short Path Distillation
STD	Standard Deviation
TAG	Triacylglycerol
TB	*Tert*-butanol (2-methyl-2-propanol)
TP	*Tert*-pentanol (2-methyl-2-butanol)
TLC	Thin Layer Chromatography
UF	Ultra Filtration
WHO	World Health Organization

List of manuscripts attached to this thesis

Articles:	Authors, Title and Journal
Paper I:	Damstrup, M. L.; Jensen, T.; Sparsø, F. V.; Kiil, S. Z.; Jensen, A. D.; Xu, X. **(2005).** Solvent Optimization for Efficient Enzymatic Monoacylglycerol Production Based on a Glycerolysis Reaction. *J. Am. Oil. Chem. Soc*. 2005, 82 (8), 559-564.
Paper II:	Damstrup, M. L.; Jensen, T.; Sparsø, F. V.; Kiil, S. Z.; Jensen, A. D.; Xu, X. **(2006).** Production of Heat-Sensitive Monoacylglycerols by Enzymatic Glycerolysis in *Tert*-Pentanol: Process Optimization by Response Surface Methodology. *J. Am. Oil. Chem. Soc.* 2006, 83 (1), 27-33.
Paper III:	Damstrup, M. L.; Abildskov, J.; Kiil, S.; Jensen, A. D.; Sparsø, F. V.; Xu, X. **(2006).** Evaluation of Binary Solvent Mixtures for Efficient Monoacylglycerol Production by Continuous Enzymatic Glycerolysis. *J. Agric. Food Chem.* 54 (19), 7113-7119.
Paper IV:	Damstrup, M. L.; Kiil, S.; Jensen, A. D.; Sparsø, F. V.; Xu, X. **(2007).** Process Development of Continuous Glycerolysis in an Immobilized Enzyme Packed Reactor for Industrial Monoacylglycerol Production. *J. Agric. Food Chem.*, 55 (19), 7786-7792.

Appendices:	Title
Appendix I:	Description of the TLC-FIDs applicability to measure compound distribution after enzymatic glycerolysis of vegetable oils and comparison to GC-FID analyses.
Appendix II:	Reaction kinetics of the enzymatic glycerolysis of vegetable oils and glycerol and sn-specificity
Appendix III:	Product Data Sheet Novozym®435.

Other publications related to the Ph.d project

Prabhavathi Devi, B. L. A.; Zhang, H.; **Damstrup**, M. L.; Guo, Z.; Zhang, L.; Lue, B-M.; Xu., X. **(2008).** Enzymatic synthesis of Designer Lipids. *Journal francais des Oléagineux, Corp Gras, Lipides* (OCL). 15 (3), mai-juin.

Xu, X.; Guo, Z.; Zhang, H. ; Vikbjerg, A. F.; **Damstrup,** M. **(2006).** Chemical and enzymatic interesterification in lipid modification. In *Modifying Lipids for Use in Foods*, ed. Gunstone, F. D. CRC Press, Cambridge, England, 234-272.

Damstrup, M.L., A. Jensen, S. Kill, F. V. Sparsø, and X. Xu **(2005).** Enzymatisk produktion af monoglycerider. *Dansk Kemi.* 86 (8), 27-30.

INTRODUCTION

1.1 Project background

Monoacylglycerols (MAG), or in a more common term expressed as monoglycerides, are minor components in natural animal fat and plant oils. In the human body, MAG is a digestion product from food rich in oils and fats, participating in essential fat-metabolism (Mu & Høy, 2004). MAGs are commercially produced from low cost fats and oils, which makes them available at reasonable prices. MAGs of edible fatty acids, either in pure form or in combination with diacylglycerol (DAG), are approved by the EU as food grade additives with EU number E471. They have GRAS (Generally Recognized as Safe) status by the FDA (U.S Food and Drug Administration), and can be used *quantis satis* (no maximum level permitted is specified) according to the European Directive (OOPEC, 2004). According to WHO (World Health Organization) and the EU directive, MAG and DAG mixtures of fatty acids are required to contain at least 70wt% MAG +DAG, at least 30wt% MAG, and maximum 7wt% glycerol (EFEMA, 2004; OOPEC, 2004). MAGs are chemically characterized by an amphiphilic molecular structure, which combines a hydrophilic and a hydrophobic portion. This gives the capability to function as emulsifiers, binding water- and non-soluble compounds but also other useful properties. MAGs beneficial combination of dietary safety, low costs and many functional properties are comercially utilized in varied fields such as in the pharmaceutical, food-, cosmetic-, and plastic industry. In the food grade area, purified MAGs and mixtures with DAGs contribute extensively with 75% to the worldwide market for emulsifying agents. This corresponds to a production of approximately 200,000-250,000 metric tons per year, which makes purified MAG and MAG + DAG mixtures to commercial important products (Krog, 1997; Moonen & Bas, 2004, Kaewthong & H-Kittikun, 2004, Danisco A/S, 2008, Boyle, 1997).

Highly pure MAG products are most widely utilized because of better functionalities than in mixtures with DAG. MAG function as surfactant in emulsions, foams, aerosols and suspensions, complex starch, interact with protein and modify fat crystallization and the viscosity characteristics. Furthermore MAGs enable control of both foaming and anti-foaming effects, dispersion of solids in water and lubrication. (Premlal Ranjith & Wijewardene, 2006; Boyle, 1997; Danisco A/S, 2008). The main

application of MAG in foods are typically in fat-based products such as margarine, spreads, bakery products, cake mixtures and confectionary owing to their emulsifying, stabilizing, and conditioning properties (Kaewthong & H-Kittikun, 2004; Krog, 1997). Examples of functionalities are as anti-sticking agents in pasta, instant mashed potatoes and cereals and anti-staling agents - or crumb softeners - in bread. By this antistaling or crumb softening effect the MAG interact with the starch amylase and hereby prevent starch retrogradation (staling). This functionality is one of the major applications of the purified MAGs (Krog, 1997, Boyle, 1997). They stabilise emulsions in margarine, destabilise ice cream and can replace unhealthy *trans* fat. MAG (+ DAG) are often added to industrial food formulations in combination with other more hydrophilic emulsifiers, for instance in combination with hydrocolloids in dairy emulsions such as ice cream (Krog, 1997). MAGs are important in cosmetic and pharmaceutical industries as drug carriers and for consistency improvements of creams and lotions. Owing to their lubricating and plasticizing properties, MAGs are used in textile and fibre processing, and production of plastics. MAGs are for instance used as anti-stat and anti-fog agents in particularly food wrap and other plastic packaging for the food industry. (Danisco A/S, 2008; Bellot *et al,* 2001; Elfmann-Börjesson & Härröd, 1999; Ferreira-Dias *et al*, 2001; Kaewthong *et al*, 2005; Yang *et al.,* 2005a).

Danisco A/S is a well established key player on the global emulsifier market that started with emulsifier production in the thirties and has produced distilled MAGs since 1964. Today, Danisco A/S has a leading market position of commercially available MAGs mixed with DAGs (GRINDSTED®MONO-DI) as well as highly pure MAGs (DIMODAN®) (Krog, 1997, Danisco A/S, 2008). Today, chemical glycerolysis of fats or oils and glycerol is a well known industrial production method to synthesize MAGs. By this glycerolysis reaction, fatty acids (FA) from the triacylglycerol (TAG) oils or fats are exchanged to the free glycerol, providing a product mixture of MAGs and DAGs. From here, MAGs can be purified by steam stripping and distillation processing. This glycerolysis process is currently performed in the presence of an alkaline catalyst at high temperature. Although this conventional process is rather efficient it suffers from one major drawback. The use of a high temperature (of 220-260°C) accelerate the development of off flavors and dark color and easily damage heat sensitive nutritional important polyunsaturated fatty acids

(PUFAs) present in the oil. This is due to easily oxidation, peroxidation and polymerization at the high temperatures. Thus, today's emulsifier market is dominated by MAGs product characterized by partially or completely saturated FA profiles (Bornscheuer, 1995; Boyle, 1997; Krog 1997; Xu, 2000; Elfmann-Börjesson & Härröd, 1999; Ferreira-Dias *et al*, 2001; Kaewthong & Kittikun, 2004).

However, a continuously growing consumer awareness of health and well being and increasingly focus on 'healthy lipids' has made an interest for industrial production of healthier MAG products. It is well known that food rich in n-3 PUFAs that contains long chain fatty acids (FA) of minimum 18 carbons have many health benefits. Anyhow, the recommended n-3 PUFA intake from governmental authorities such as WHO, FDA and AHA (American Health Association) of 1-2 g/day are in general not fulfilled in western diet (Li *et al.,* 2003; Hornstra, 2004; Sioen *et al.,* 2007; Gebauer *et al.,* 2005). Therefore, the n-3 PUFA intake should be increased beyond levels currently consumed. Since purified MAGs are widely used in industrial processed food-products, addition of 'nutrional improved' MAGs can be an easy way to enhance the intake of healthy n-3 PUFAs without any dramatically changes in consumers eating habits. Therefore, health improved MAGs are believed to have a high potential in balancing the types of fats in the diet according to common recommendations. By replacing today's mainly saturated MAGs with MAGs containing essential unsaturated FA the way for making functional additives/ healthier emulsifying agents can be adressed.

Introduction of biotechnology, using enzymes as biocatalysts is of main importance for making production of such health improved fats and fat deviates feasible. Since enzymes are highly active at 'ambient' temperatures (less than 80°C) mild reaction conditions can be employed to avoid heat accelerated damage of the heat sensitive unsaturated FA structure. The first generation of enzyme catalyzed fats and oil bioprocesses are already implemented into commercial plants with health improved lipids availble on the global market. The Enova^{TM} DAG cooking oil, produced from patented Lipozyme RM-IM catalyzed esterification of fatty acids from soy and canola oils and the human milk fat substitute Betapol^{TM} produced by lipase catalyzed interesterification of vegetable oils are examples of that. With more than 160 million bottles Enova^{TM} sold (2008), being Japans most selling cooking oil at the

moment, having a widespread distribution in the U.S and is on its way to Europe, it strongly emphazises the great potential of commercial utilized bioprocesses (Flickinger & Matsou, 2003; Matsou, 2005; Akoh, 2005; Enovaoil, 2008).

Lipase catalyzed glycerolysis is an extensively studied method capable of handling unsaturated fats and oils at ambient temperatures to produce healthful MAGs. Although several laboratory scale investigations confirms the great potential of this method it has still not led to a enzyme catalyzed process that replaces or supplement the current used industrial chemical glycerolysis. The very poor miscibility of the glycerol and oil at low temperatures in combination with a very high reactant viscosity results in a reaction system with high mass transfer limitations. As a result long reaction times, and/or limited conversion to MAG in general make the enzymatic reaction inefficient for the industry (Elfmann-Borjessön & Härröd, 1999; Ferreira-Dias *et al*., 2001; Kaewthong & H-Kittikun, 2004: Coteron *et al*., 1998; Rosu *et al.,* 1997). Hence, one of the main challenges by using enzymatic glycerolysis is the complexity of the heterogeneous multiphase system comprising of three phases: Solid enzyme particles, hydrophilic glycerol and lipophilic oil. Recently, using solvents in the enzymatic glycerolysis seems to overcome the immiscibility of the glycerol and oil and to improve the contact to the lipase enzyme. From applied point of view, the use of assistance media and immobilized enzyme packed bed reactors is therefore considered as very potential approaches to commercialize the glycerolysis process on an industrial scale (Yang *et al*., 2005a; Goto *et. al.,* 2005; Garcia *et al.,* 2001, Bellot *et al.,* 2001; Murty *et al*., 2005; Moreno *et al.,* 2005).

Hence, time has come for industry to careful considers implementation of an industrial applied bioprocess for MAG production to provide consumers with health enhanced emulsifying agents in coming years. Furthermore, by applying lipases from nature to industrial production a ‘white biotechnology’ or ’industrial biotechnolgy’, characterized by a safe and sustainable bioprocess, becomes feasible.

1.2 Purpose of the project and thesis outline

Process- and product-development is of great importance to maintain market position and to fulfill the continuously changing consumer demands. For Danisco A/S, it is important to maintain the position as leading MAG manufacturer on an important food emulsifier niche market. Hence Danisco A/S has requested an in-depth investigation of the possibilities for applying enzymatic glycerolysis in industrial plant to produce purified MAGs. Danisco A/S is seeking an industrial production method that is capable of handling heat sensitive fats and oils with PUFAs, especially n-3 PUFAs. By developing a supplementary industrial applicable bio-process at ambient temperatures it allows for the expansion of the MAG product port folio and potentially for more valuable products.

A cooperative pre-project between Danisco A/S and BioCentrum-DTU from 2002 investigated the enzymatic glycerolysis conducted in a *tert*-butanol (TB) medium for MAG and DAG production and its potential for industrial utilization (Yang & Xu, 2003). The pre-project confirmed a great industrial potential of enzymatic glycerolysis and provided a lot of useful answers. However, it also raised some new questions, which had to be investigated before a scale-up/industrial application was realistic. Therefore, the present series of studies were initiated to obtain a better understanding of the potential glycerolysis route and the industrial applications. Using TB in the system solved the problem with the immiscibility of the glycerol and oil raw material and reduced the reactant viscosity. Hence, it succeeded to develop an efficient reaction with high MAG formation in short time (Yang & Xu, 2003). However, from a practical point of view TB is difficult to handle owing to crystallization at room temperature. In addition, a narrow temperature range between the m.p and b.p of TB allows a risk of solvent crystallization during condensation in the subsequent MAG purification. When crystallization occurs, it can be difficult to condense the solvent, which worsens the possibility of reuse, aspects of great importance for industrial usage. Hence, a more careful solvent evaluation was needed to include and compare physical properties and prices with the effect on the reaction system. In the pre-project, a lipase screening showed superior functionality of the

comercially available Novozym®435 in the solvent glycerolysis system (Yang & Xu, 2003). Thus, a more in-depht evaluation of this enzyme was desirable to evaluate the cost, stability, specificity, capacity, and mechanism in the actual set up. A better understanding of the mass transfer phenomena in continuous column reactors was required. The pre-project dealt mainly with the MAG synthesis, therefore further considerations about the subsequent purification were needed to obtain a full implemented process line. Finally, up-scale considerations that further developed on the suggested continuous columns reactor was necessary to take typically up-scale problems such as pressure drops into account. The aim for this Ph.D. study can therefore be summarized to combine progressing research in laboratory scale with practical oriented aspects to access the large scaled industrial enzymatic glycerolysis. In more details the aims for the present Ph.D. thesis is to:

- Develop a continuously operated and efficient production line for MAG production feasible for implementation in industrial facilities, in which MAG synthesis by lipase-catalyzed glycerolysis and subsequent purification are included.
- Design the process with respect to handling of lipid components carrying nutritional important PUFAs sensitive to oxidation, heat etc.
- Conduct a careful evaluation of organic media suitable for enzymatic glycerolysis which includes comparison of the polarity, physical properties and prices towards the reaction performance and practical feasibility.
- Investigate the sn-regio specificity, stability and capacity of the Novozym®435 enzyme and evaluate the mechanism of the glycerolysis reaction catalyzed by this enzyme.
- Evaluate plausible mass transfer phenomena in an enzyme packed column reactor.
- Consider an up-scale process from investigations in a pilot plant facility.
- Elaborate on the sustainability of the developed process and the advantages/disadvantages compared to the chemical glycerolysis.

1.3 Thesis approach

The Ph.D. study behind this thesis was approached from an industrial applied point of view and was done within the area of lipid – and enzyme technology and chemical engineering. The work included a literature study, laboratory and pilot plant experiments and result evaluation within following four defined research- and working areas:

- Analytical issues centered on the TLC-FID methodology
- MAG synthesis through the enzymatic glycerolysis reaction route
- MAG purification considerering distillation techniques to other methods
- Up-scaled experiments in a pilot plant facility with enzyme packed column reactors.

The present thesis reports and summarizes the main findings from this work. The thesis comprises of a theoretical introduction to the subject followed by a condensed describtion and discussion of the substantial experiments conducted. Some issues are elaborated in further details in four published articles and two appendices, referred to in the text by Paper I-IV and Appendix I-II. An overview of these manuscripts is given in the front of this thesis and the actual manuscripts are attached in the back.

LITERATURE STUDY

2. Properties and functionalities of MAG

2.1 Chemical characteristics

MAG consist of a single fatty acyl chain esterified (-COO-) to a glycerol (gly) backbone and comprise together with DAG (that contains two FAs) the compounds often termed 'partial acylglycerols'. They are non-ionic, amphillic molecules with the free hydroxyl groups (OH) of the glycerol providing hydrophillic characteristics, while the acyl chain (RCO) provides lipophilic characteristics (Fig 2.1).

.

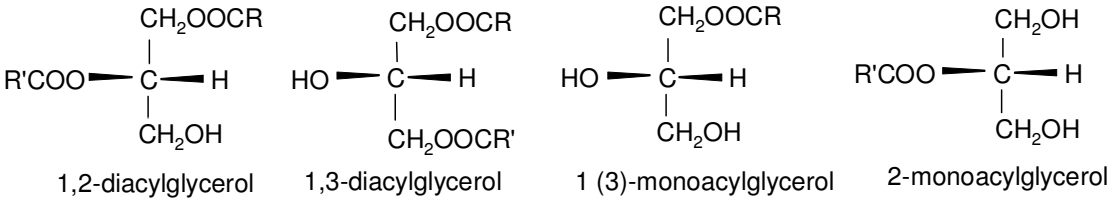


Figure 2.1: Illustration of the chemical structure of different stereo isomeric partial acylglycerols. R represents a fatty acid residue.

The individual fatty acids can be located on different carbons of glycerol, numbered sn-1(3) for the end carbon position and sn-2 for the middle carbon position. The esters are called α-isomer on primary and β-isomer on secondary OH group (O'keefe, 1998). Depending on the lipid origin, the FA can in principle obtain all kinds of fatty acids: Saturated, unsaturated, long chained or short chained. Fatty acids with acyl chain lengths of 10-20 carbons (especially 16-18) are the most common for chemically synthesized MAGs (Boyle, 1997, McClements, 1998).

2.2 Common isomeric forms and acyl migration

The length, unsaturation and branching degree of the FA influences to a certain extent the distribution of the different MAG isomeric forms. Anyhow, the most stable sn-regio form of MAG is in general the sn-1 position. At equilibrium conditions chemically synthesized long chained MAGs typically reaches a 1-MAG:2-MAG ratio of ~ 9:1 This ratio is formed due to spontaneous intra molecular thermodynamically induced reactions known as acyl migration (Laszlo *et al.,* 2008,

Millqvist *et al.*, 1996). By acyl migration, the FA groups attached to the sn-2 position can spontaneous invert to the sn-1 position, proposed by a mechanism illustrated in Fig. 2.2.

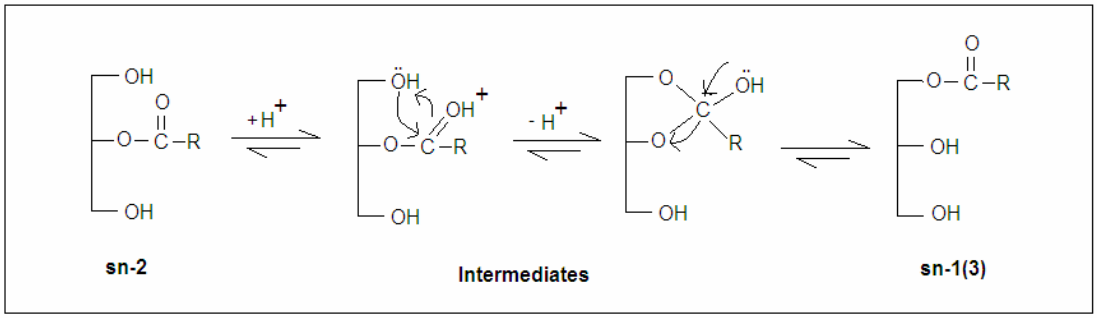


Figure 2.2: Proposed mechanism for acyl migration. Adapted from Millqvist et al. (1996).

In the first step of the acyl migration, the presence of a proton (H^+) enhances the electrophillic character of the second postioned carbonyl carbon, which is then nucleophillic attacked by a primary hydroxyl oxygen (Ö). This results in a five membered ring intermediate, an unstable orthoester. In the last step, the hydroxyl oxygen again makes a nucleophilic attack on the carbon which results in a cyclic intermediate disrupt. The ring is hereby opened and the more thermodynamically stable isomers of unbranched 1-MAG are formed (Millqvist *et al.*, 1996; Laszlo *et al*, 2008; Peng *et al.*, 2000).

2.3 Nutrition and health effect

The nutritional values of acylglycerols are as carriers of essential FA and as energy sources for the human body. The acylglycerols, predominantly ingested in the form of TAG, are degraded to gly, free fatty acids (FFA) or 2-MAGs in the stomach by several lipases before being absorbed in the gastrointestinal system. After absorption the lipid components participate in re-synthesis of TAG and other important metabolic reactions (Mu & Høy, 2004). Of the initially ingested TAG, approximately 75-80% is hydrolyzed to 2-MAGs, absorbed and rapid re-synthesized to TAG (Boyle & German, 1996; Mu & Høy, 2004). Hence, from a nutritional point of view, sn-2 MAGs are of great importance for directly absorption in the intestinal system. 1-MAGs are typically hydrolysed to FFA and gly that also participate in re-synthesis reactions just by another reaction route (Mu & Høy, 2004). Thus, other

partial acylglycerols are nutritional important as well. Different sn-regio specific MAG components can therefore all contribute with a nutritional value as long as the FAs are essential. Linoleic acid C18:2 (n-6) and linolenic acid C18:3 (n-3), being converted to desaturated and elongated metabolic active components (C20:4n-6 and C22:6n-3) are some of the most common essential n-3 and n-6 long chained PUFAs supplied to the body by our diet (O'Keefe, 1998).

Dietary lipids and lipid deviates assume many phases throughout its metabolism in the body. These phases include emulsion, micelles, lamellar, cubic and hexagonal (Fig 2.2), all of which are necessary for the reactions essential to fat metabolism (Boyle and Garmen, 1996, McClements, 1998).

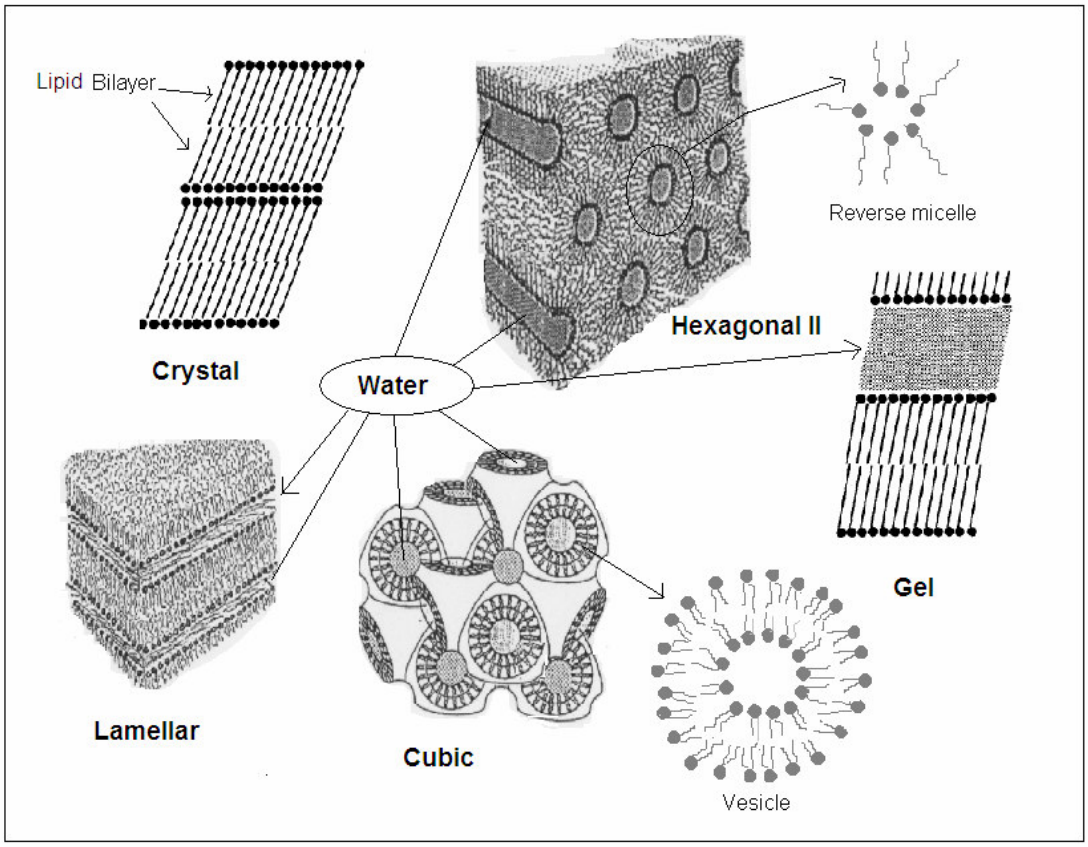


Figure 2.2: Illustration of some of the structures known as association colloids (aggregation of amphilic molecules) and mesophases (colloids interaction with water) that are frequently formed by amphilic molecules (MAG and DAG) through metabolism reactions. The figure is partly adapted from Danisco A/S and partly from McClement, 1998. represents polar head, represents apolar tail.

The necessity of these various lipid phases is twofold. Formation of these aggregation structures (known as association colloids) allows the fat to coexist in a water system (termed mesophases). Furthermore, many of the enzymes present in the

absorption pathway have a requirement for the lipid substrate being accessible in a specific physical form. Enzymes are for instance typically inactive on monomer lipid molecules but highly active when the lipids are formed in structured bilayers (Boyle & Garmen, 1996).

2.4 Colloidal aspects and physical properties

The balance of hydrophobic and hydrophilic portions allows MAGs to function as surfactants, reducing the interfacial tension in a region of 'water'- and 'oil'-liking phases. The surfactants molecules are dispersed predominantly as monomers which helps to unite two immiscible phases in emulsions (one mixture dispersed into another), foams (gas/air bobbles trapped in a liquid or solid), aerosoles (liquid/solids in air/gas) and suspensions (solid particles dispersed into a liquid) (Boyle, 1997; McClements, 1998; Premlal Ranjith & Wijewardene, 2006).

In solutions, at reasonable concentrations (above the critical micelle concentration), MAGs and other lipid-based amphiphlic molecules aggregate spontaneously to form a variety of thermodynamically stable structures. These association colloids include for instance lipid-bilayer, micelles, reverse micelles and vesicles similar to the ones formed throughout human fat metabolism (Fig. 2.2). In many food emulsions, surfactant micelles are the most important type of association colloid formed (McClements, 1998). By these colloidal formations the unfavorable contact area between the nonpolar tails and the hydrophilic head is minimized. The forces holding association colloids together are relatively weak why they have highly dynamic and flexible structure. Their size and shape is continually fluctuating and individual molecules rapidly exchange between micelle and monomers in the surrounding liquid. Despite this highly dynamic nature, the associate colloids do form well-defined average size (McClements, 1998). In general, colloids roughly have dimensions between 10^{-9} and 10^{-6} m (in one direction) (Iupac, 2008). Thus when MAGs and other surfactants is added to a solution above a critical amount, the number of micelles increases, rather than the size (McClements, 1998).

Due to the relatively high amounts of 'hydrophobic' FA in MAG + DAG mixtures, dispersion in water is rather limited (Krog, 1997). However, MAGs have better dispersibility in water, making pure MAGs capable of interacting with water in well defined structures, known as mesophases (Krog, 1997; Boyle, 1997). This unique behavior of MAG in water results in different physical structures at different conditions (Fig 2.3) such as temperature, MAG and water concentration, FA chain length, saturation degree, and sn-isomeric position of the FA (Krog, 1979, Boyle, 1997, Boyle & German, 1996; McClements, 1998; Krog, 1997).

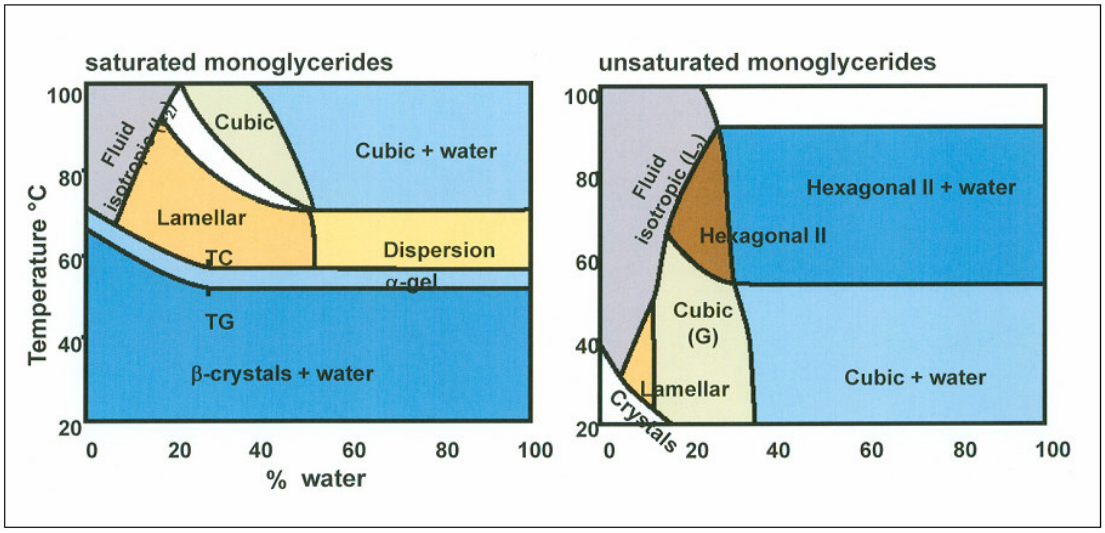


Figure 2.3: Phase behaviour of pure MAGS at varied temperatures and water contents. Adapted from Danisco A/S.

The distinct mesophases of MAGs: Crystal, fluid isotrop, lamellar, gel, cubic, hexagonal, and dispersion (Fig. 2.3) differ in both MAG packing, association to water and physical properties. In the crystal structure (Fig. 2.2), which have been reported for 2-monolaurin, MAGs are packed head to head and tail to tail in repeating layers. The crystal are 'solids' and can be found in the α-, β-, or β'- polymorphs form, with β being most stable. The fluid isotropic structure is a loosely packed structure and exhibit random fluid characteristics similar to thin oil. The lamellar mesophases is structured by a lipid bilayer separated by water layers (Fig 2.2) that can be up to several hundred angstroms in thickness (10^{-8} m) (Krog, 1997). The lamellar structure has a long-range order but no short-range order similar to the crystalline phase (like fats) and is also known as liquid crystals. The lamellar structure is reported for MAGs such as monopalmitin and monocaprylin and monoolein. It appears that saturated MAGs are able to incorporate more water in the lamellar phase than unsaturated fatty

acids, which might be due to altered packing induced by the double bonds. The gel phase is structured by a continuous bimolecular MAG layer similar to the lamellar phase (Fig. 2.2). The main difference lies in the hydrocarbon being ordered and a greater lipid thickness. At high temperatures a viscous isotropic phase known as the cubic phase occurs. The cubic phase is structured in a three-dimensional continuous lipid bilayer separated by water channel systems (Fig. 2.2), in which other hydrophilic components may be capable of being incorporated (Boyle & German, 1996). The hexagonal phase occurs at high temperatures in some MAG systems, structured in continuous bilayers, with the polar headgroup surrounding and separating water channels by reverse micelles (Fig 2.2). The dispersion phase is formed from the lamellar phase at high water content. Here, MAGs are dispersed in water and contain water themselves. Dispersed MAGs are structured like lamellar but differ by spherically symmetric particles (Boyle and German, 1996)

Because of these different mesophases structures MAGs exhibit varied functionalities in addition to their emulsifying properties. They can function as for instance starch complexers, interact with protein and modify fat crystallization and the viscosity characteristics. They enable control of both foaming and anti-foaming effects, the dispersion of solids in water and lubrication (Premlal Ranjith & Wijewardene, 2006; Boyle, 1997; Danisco A/S, 2008). For aeration functions, such as whipping creams, the MAGs should for instance be maintained in a liquid crystalline structure (Boyle, 1997). The gel phase is an important physical state for the texturing agents in fat-free foods, owing to the increase in the viscosity (Krog, 1997). Most food emulsions are rather complex with an aqueous phase that may contain water soluble ingredients of many kinds and a oil phase that most likely contain a varity of lipid-soluble components (McClements, 1998). For instance, addition of sugar promotes the transition of MAGs and water from the cubic phase into a reverse hexogonal phase (Boyle & German, 1996). Hence, MAGs functionalities are individual depending on the food-system in which they occur.

3. Industrial production of MAGs

A conventional and well-known industrial utilized strategy to produce MAGs (+ DAGs) is by chemical glycerolysis followed by subsequent MAG purification. The capability of utilizing cheap TAG fats and oils sources (of animal and vegetable origin) and a highly efficient production way makes this reaction route very attractive for industry. The purification involves vacuum distillation techniques from were the term 'distilled MAG' products arises (MAGs in a highly pure form) (Danisco A/S, 2008). An overview of an industrial employed process line for destilled MAG production is illustrated in Figure 3.1.

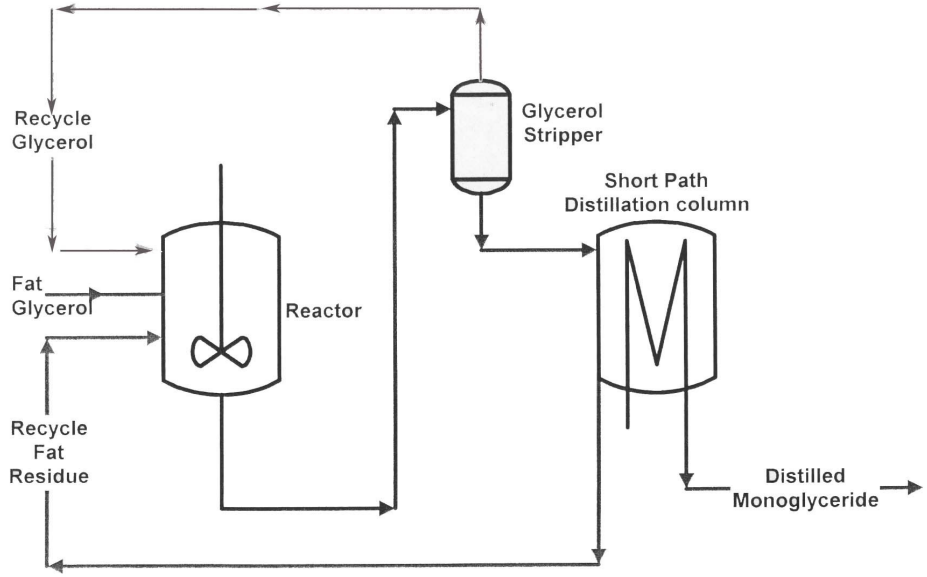


Figure 3.1: Illustration of the principle sketch of the production of distilled MAGs in an industrial plant. Adapted from Danisco A/S.

3.1 Chemical glycerolysis

Chemical glycerolysis is the reaction between glycerol and fats or oils, chemically characterized by a TAG molecular structure. By using an inorganic alkaline catalysts, such as NaOH or $Ca(OH)_2$, and temperatures of approximately 220-260°C acyl groups (R-C=O-) are transferred to form a product mixture dominated by MAGs and DAGs (Fig. 3.2) (Bellot *et al.,* 2001; Bornscheuer, 1995; Elfmann-Borjessön & Härröd, 1999; Kaewthong *et al*., 2005; Krog, 1997; Lee *et al.,* 2004; Rosu *et al*., 1997).

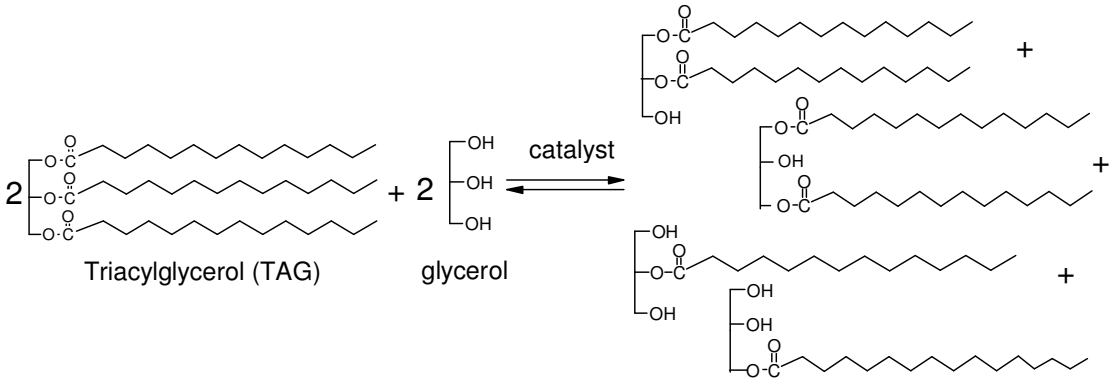


Figure 3.2 Chemical characterization of the glycerolysis reaction in which fatty acids from the triglyceride are transferred to the free glycerol to form a varity of acylglycerols.

The glycerolysis reaction is a specific situation of the 'alcoholysis' reaction in which a TAG ester bond is cleaved to produce a new ester with one of the glycerols alcohol moieties (-OH) (Peng *et al.,* 2000; Xu, 2003). Owing to the involvement of ester cleavages as well as ester formations the reaction is sometimes termed inter-or transesterification (Xu, 2003; Malcata *et al,* 1992). Although, the transferred acyl moieties is randomly distributed, the sn-1 regio specific MAGs and sn-1,3 DAGs clearly dominates (Krog, 1997). This is due to spontonuously acyl-migration from the sn-2 position to the sn-1 position by the mechanism described in paragraph 2.2. The glycerolysis reaction can to a certain extent favor the formation of certain acylglycerols (MAG or DAG), by adjusting the reaction conditions such as glycerol to oil ratio, reaction time and pressure (Peng *et al.,* 2000). Current chemical glycerolysis processing is usually conducted with a relatively low molar ratio of glycerol to oil of about two (Rendon *et al*., 2001). This provides a distribution between MAG, DAG and TAG of 45-55%, 38-45%, and 8-12%, respectively (Krog, 1997). The glycerolysis reaction is reversible. Hence, minor quantities of free glycerol and TAG are typically obtained in the equilibrium product mixture. Furthermore, the product mixture often constitutes minor amounts of FFA (Krog, 1979).

3.2 MAG purification

Since MAGs are the active component in the glycerolysis product mixture it is natural to concentrate them in a highly pure form by removal of excess glycerol, TAG, DAG, FFA and other impurities. Industrial MAG purification includes typically

glycerol stripping in a deodorizer column and MAG concentrating by short path distillation (SPD) (Bellot *et al*., 2001; Bornscheuer, 1995; Elfmann-Borjessön & Härröd, 1999; Kaewthong & H-Kittikun, 2004; Krog, 1997; Krog, 1979; Lee *et al.,* 2004; Rosu *et al*., 1997; Xu *et al*., 2002a).

3.2.1 Glycerol removal by steam stripping in deodorizer column

Deodorization (= to neutralize the odor of) is a typical step in the oil refining process to remove compounds responsible for undesirable odors and flavors such as low molecular weighted FA, aldehydes, ketones, alcohols, peroxide decomposition products, hexane etc. The principle of deodorization is steam distillation, also known as 'steam stripping' perfomed at high temperatures (180-270°C) and vacuum conditions (3-8 mm Hg). Steam is injected into a deodorizer column to carry away or 'strip off' the volatiles and to provide agitation. The vapor is hereafter condensed in condenser and collected as distillate (Johnson, 1998; Xu *et al.,* 2002a). The principle sketch of steam stripping is illustrated in Fig 3.3

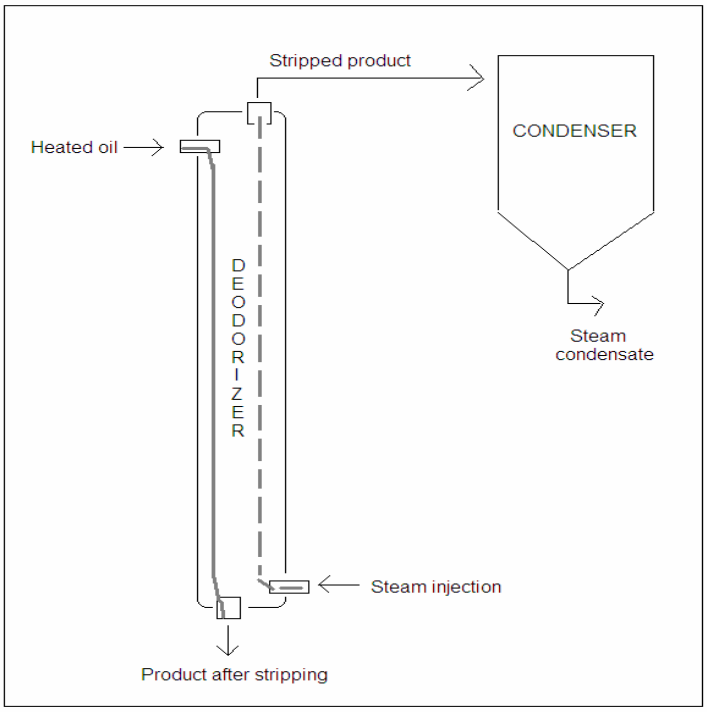


Figure 3.3: Principle sketch of the steam stripping in a deodorizer column.

Glycerol amounts up to 10 wt% is current removed from the glycerolysis mixture during chemical MAG production by steam stripping at temperatures of about 190°C and a pressure of 2-5 mbar with subsequent external condensation at 80°C (Danisco

A/S, 2008). Besides to help with improved product quality and a more pure product the steam stripping makes re-use of glycerol possible, which is beneficial to reduce the raw material costs.

3.2.2 MAG separation by short path distillation

The principle of the SPD is that MAG can be distilled from the higher boiling DAG and TAG lipids under low pressure and a short reaction time (Fig. 3.3).

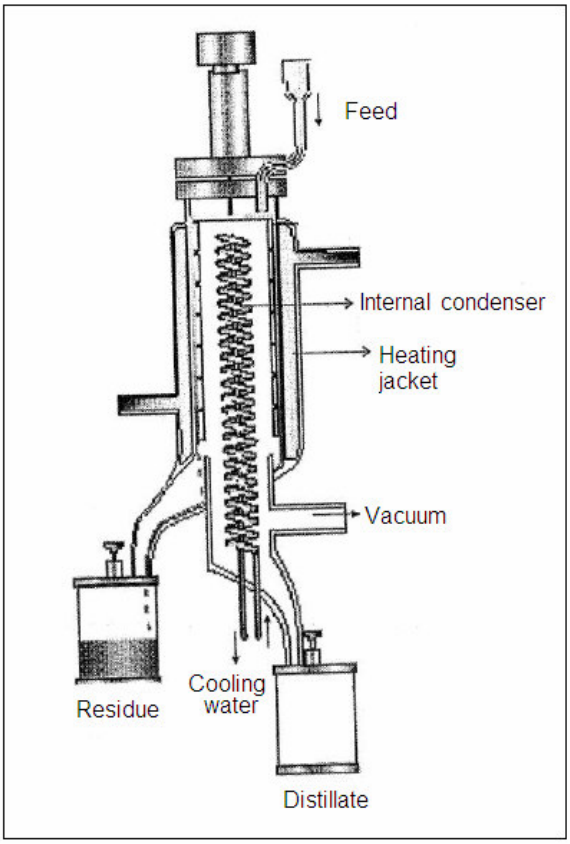


Figure 3.3: Principle sketch of the short path distillation. Adapted from Xu (2005).

The acylglycerols are feed into an evaporator under low pressure (≤ 0.1 mbar). Here the feed is immediately spread into a very thin film, and forced quickly down the evaporator surface. The heated walls and a high vacuum drive the more volatile MAG components to the closely positioned internal condenser where they are concentrated in a distillate. The less volatile DAG and TAG continue down the cylinder and are collected as the residue or retentate (Xu, 2005). The heat labile compunds are sufficiently volatile before unwanted decomposition or polymization occurs. For

glycerides containing pure saturated FA, the boiling point (b.p.) of the corresponding MAG, DAG and TAG differ by > 50°C under different vacuum conditions (Xu, 2005). A typically set up to purify MAG from glycerolysis could be MAG evaporation at 140-185°C under vacuum (<0.1 mbar) for 1½-2 minutes followed by condensation (Danisco A/S, 2008; Xu, 2005). After SPD highly pure MAG with a very low content of DAG of less than 4wt% and only traces of FFA and glycerol are obtained (Krog, 1979).

4. Enzymatic glycerolysis

Lipases constitute a very important group of biocatalysts for biotechnological applications owing to high-level production from microorganism, a detailed understanding of the molecular mechanism and established novel utilizations (Jaeger & Eggert, 2002; Reetz, 2002). In enzymatic glycerolysis, lipases functionalities as biocatalysts offer a variety of benefits that can improve the process and product quality. Lipases are highly active at ambient temperatures which reduce some of the common heat accelerated problems arising from chemical catalyzed glycerolysis. By lowering the temperature potentially oxidation, peroxidation and polymerization is diminished. Hence, development of bad flavor, dark-colored by-products, burnt taste and easily damage of sensitive unsaturated FA profiles are minimized. In addition, avoiding the extreme temperatures and metallic catalysts reduces the energy costs and catalyst waste, both of environmentally importance. Furthermore, enzymes acts in general specific which opens the way to produce 'new' specific designed partial acylglycerols (McNeill & Yamane, 1991; Elfmann-Borjessön & Härröd, 1999; Kaewthong & H-Kittikun, 2004; Rosu *et al*., 1997; Peng *et al.,* 2000). The enzymatic glycerolysis is therefore considered as a very potential alternative to chemical glycerolysis.

4.1 Action of lipases

Lipases are characterized as TAG hydrolases and displays in general maximum activity at pH values ranging from 5.6 to 8.5 and a temperature between 30

and 40°C (Malcata *et al.,* 1992). They are unique enzymes in their preferences for hydrophobic substrates. However, lipases have almost no activity with lipid substrates in a monomeric form. Water is essential for enzyme activity and plays an important role in maintaining the enzymes native conformation and enzyme dynamics. Hence, a characteristic feature of lipases is their activation at the water/lipid interfaces for full catalytic performance (Fukunaga *et al.,* 2002, Martinelle *et al.*, 1995; Goto *et al.,* 1995; Martienlle & Hult, 1995; Zaks, 1991). A common feature among lipases is a protein structure with an active triad Ser-His-Asp site protected by a polypeptide 'flap' or 'lid'. The activation phenomenon in the interface involves conformational change in which the lid moves to uncover the active site. By this 'lid' regulated accessible active site a general high degree of substrate and stereo specificity are obtained (Fig. 4.1).

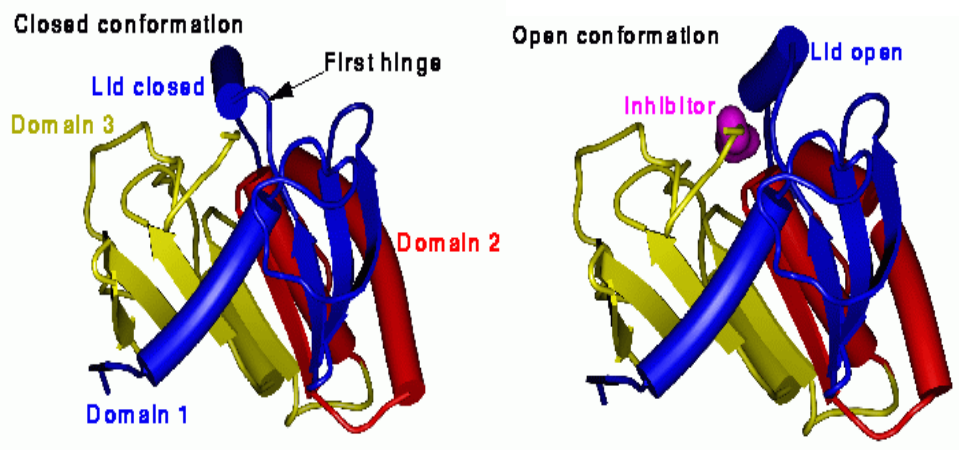


Figure 4.1: Illustration of the lipase 3D structure and the conformational change during activation. Adapted from: http://www-bioeng.ucsd.edu/~herrgard/activation.html, 2008)

In the 'open' conformation a number of hydrophobic lipase residues are exposed which attracks lipid substrates such as TAG, DAG, MAG or FFAs (Uppenberg *et al.,* 1994; Grochulski *et al.,* 1993). By that the lipase can take action on an estergroup of a lipid substrate. The acylgroup is cleaved of, bonded to the lipase and hereafter esterified to avaible alcohols or waters (Malcata *et al.,* 1992; Fureby, 1995; Peng *et al.*, 2000). This lipase acylation mechanism and the reversal are detailed illustrated in Fig 4.2.

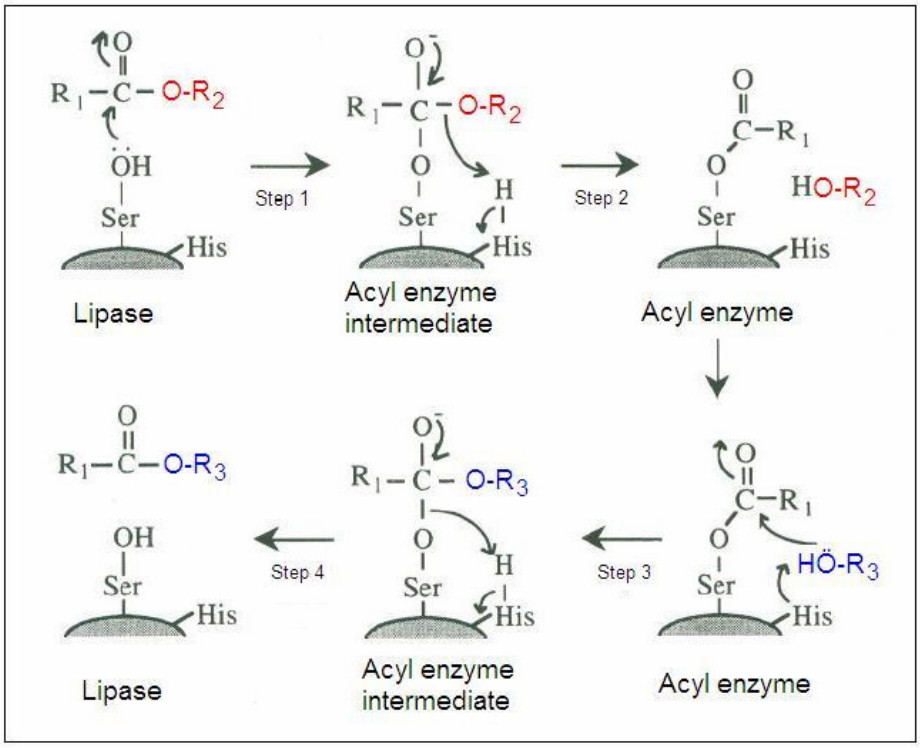


Figure 4.2: Proposed mechanism for lipases catalytic action. Adapted from Fureby, 1995.

First, the hydroxyl oxygen (Ö) from the serine chain of the lipase makes a nucleophillic attack on a carbonyl carbon on the lipid substrate (R_1-COO-R_2) resulting in an acyl enzyme intermediate. The hydrogen (H) from the serine hydroxyl group is then temporarily transferred to the lipases histidine residue placed nearby (Step 1, Fig. 4.2). The formed intermediate then covalently ester link an acyl-group (R_1-C=O-) to the serine group of the lipase (termed acyl enzyme). Furthermore, the hydrogen from the histidine is transferred to the alcohol moiety of the substrate (-O-R_2) to form an alcohol (HO-R_2) which is released from the lipase (Step 2, Fig. 4.2). Step 1 and 2 constitute the 'hydrolysis/alcoholysis reaction', termed after the product released (water or alcohol). Secondly, the hydroxyl oxygen (Ö) from another alcohol or water (HO-R_3) makes a nucleophillic attack on the carbonyl carbon bonded to the serine on the lipase. From that another 'new' acyl enzyme intermediate is formed (Step 3, Fig 4.2). This intermediate again rearranges and a new ester is released (R_1-COO-R_3) (Step 4, Fig. 4.2). Step 3 and 4 are common termed 'esterification' or 'ester synthesis' after the formed ester-product (Fureby, 1995; Malcata *et al.,* 1992; Peng *et al.,* 2000).

4.1.1 Enzymatic glycerolysis reaction mechanism/kinetics

Kinetic studies using lipases has become important to understand and explain the mechanism behind the lipase-catalysed reactions and to optimize these systems (Moquin *et al.*, 2006; Arcos *et al.,* 2001; Cheirsilp *et al.*, 2007). The most generally accepted lipase-catalyzed reaction kinetics is the so-called 'ping-pong bi-bi mechanism' which also counts the glycerolysis reaction (Bousquet-Dubouch *et al.,* 2001, Arcos *et al.,* 2001; Cheirsilp *et al.,* 2007). The kinetical equation behind this mechanism is illustrated in Fig. 4.3

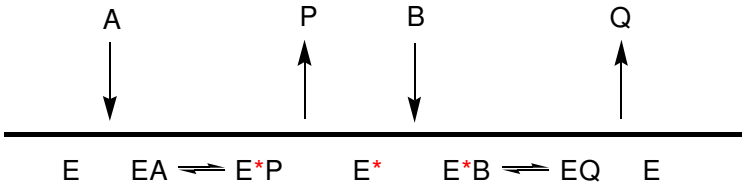


Figure 4.3: Illustration of the bi-bi ping-pong mechanism with bi(=two) substrates (A+ B) and bi(=two) products (P+ Q). A=TAG, B=gly, E=enzyme, P=DAG and Q=MAG.

Bi-bi specifies the number of reactant and products (bi=two) and the ping-pong effect indicates that one product must be released before the second substrate can bind to the enzyme. For the glycerolysis reaction that means that TAG (reactant A) initially is attached to the lipase (E). Here it is cleaved to an acyl-enzyme intermediate (E*) and DAG (product P) being released in its free form. Subsequently, the glycerol (reactant B) is attached to the enzyme and esterbonded to the acyl-enzyme intermediate (E*B) to form MAGs (EQ), relased from the lipase (Q). The lipase (E) carries out the bi-bi ping pong mechanism by the principle illustrated in Fig 4.2 (Fureby, 1995; Peng *et al.,* 2000).

Recently, more concurrent sequential reactions have been suggested to be involved in the reaction kinetics describing the enzymatic glycerolysis. These covers alcoholysis (oil + alcohol) (Eq. 4.1-4.2), hydrolysis (oil + water) (Eq. 4.4- 4.6), interesterification (ester-ester exchange) (Eq. 4.3) and the reverse reactions + acyl migration/isomerisation (described in paragraph 2.2). (Cheirsilp *et* al, 2007; Tan & Yin, 2005; Moquin *et al.*, 2005).

$$\text{TAG + gly} \underset{k2}{\overset{k1}{\rightleftarrows}} \text{DAG + MAG} \quad (4.1)$$

$$\text{DAG + gly} \underset{k4}{\overset{k3}{\rightleftarrows}} \text{2 MAG} \quad (4.2)$$

$$\text{TAG + MAG} \underset{k6}{\overset{k5}{\rightleftarrows}} \text{2 DAG} \quad (4.3)$$

$$\text{TAG + } H_2O \underset{k8}{\overset{k7}{\rightleftarrows}} \text{DAG + FFA} \quad (4.4)$$

$$\text{DAG + } H_2O \underset{k10}{\overset{k9}{\rightleftarrows}} \text{MAG + FFA} \quad (4.5)$$

$$\text{MAG + } H_2O \underset{k12}{\overset{k11}{\rightleftarrows}} \text{Gly + FFA} \quad (4.6)$$

Of these suggested kinetical equations Eq. 4.1 is believed to be the far dominating at equilibrium. Due to the hydrophilic nature of glycerol water impurities can easily be included in the glycerol reactants leading to hydrolysis (Eq. 4.4 to 4.6) (Moquin *et al.*¸ 2006). Furthermore, addition of water is common to promote the lipase activity owing to its water/lipid interfacial activation (Yang *et al.,* 2005a; Cheisilp *et al*., 2007). Spontanous acyl-migration often encounter in selective synthesis of sn regio-pure partial acylglycerols. The acyl migration rate is affected by various aspects such as presence of acids, bases, ion-exchange resins, heat and solvents (Peng *et al.,* 2000). However, even without acyl-migration considered many concurrent reactions (Eq. 4.1 to 4.6) can occur which makes the kinetical models describing the glyceolysis mechanisms complex. Hence, mathematical modelling is typically required and many parameters have to be simulated to determine reaction rates (Tan & Yin, 2005; Cheirsilp *et al.,* 2007). This modelling suffers from the risk of lack of fit and /or models that are very very limited in applicable range and conditions (Peng *et al.*, 2000; Cheirsilp *et al*., 2007). Hence, as an alternative the reaction kinetics can be approached by the use of simple and practical usable models based on the reactants and product concentrations in few selected reaction steps.

4.1.2 Characteristics of *Candida antarctica* lipase B

Candida antarctica lipase B (CALB) originating from yeast (Martinelle *et al.,*1995) is one of the lipases that have demonstrated unique and superior reaction performance in the glycerolysis reaction (Yang *et al.,* 2005a; Kristensen *et al*, 2005).

CALB is made up of 317 amino acids and has a catalytic triad of Ser105, Asp187 and His224 similar to other lipases. However, CALB deviates from the sequence around the active site of serine that is found in other lipases (Uppenberg, 1994). In CALB, a large hydrophobic surface of lipid-like molecules, most likely β-octyl glucoside, surrounds the entrance. These parts attrack monomer lipid molecules (Uppenberg, 1994). Hence, CALB displays maximal hydrolytic activity towards substrates in a water/lipid interface, but also towards monomeric lipids substrates in solutions. Thus, in contrast to many other lipases CALB is not interfacial activated and behaves more like an esterase in aqueous systems (Martinelle & Hult 1995). The lack/absence of the 'lid' that regulate the access to the active site is proposed to be the basis for this 'anomalous' behavior of CALB compared to other lipases (Martinelle *et al.*, 1995).

5. Process technology related to enzymatic MAG production

The enzymatic glycerolysis is a rather complex multiphase system because of heterogenuos components included: Hydrophillic glyceol, hydrophobic oil and solid lipase particles (Xu, 2003) as illustrated in Fig. 5.1.

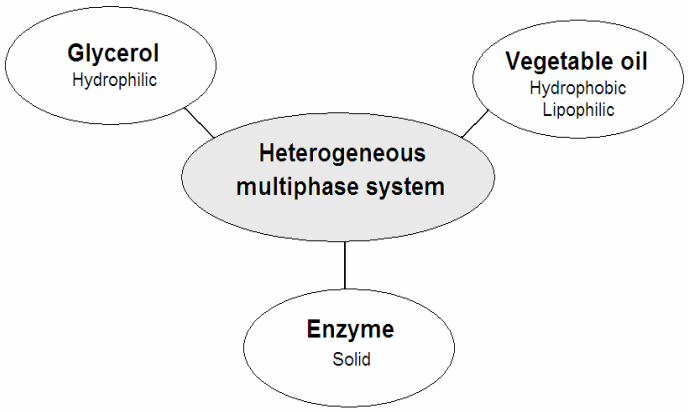


Figure 5.1: Illustration of the heterogeneous reactant mixture used for enzymatic glycerolysis.

At the ambient temperatures hydrophilic glycerol has a very low solubility in the hydrophobic fat or oil and both reactants have a very high viscosity (Kaewthong & H-Kittikun, 2004, Bellot *et al.,* 2001; Elfmann-Börjesson & Härröd, 1999). This makes mixing of the glycerol and oil into a homogenuous and stable suspension very difficult. Strong agitation, used in chemical glycerolysis can not overcome these difficulties due to enhanced risk for enzyme inactivation by conformational change of

the flexible lipase molecule by the stirring shear forces (Watanabe *et al*., 2005). Owing to the hydrophilic characteristics of the lipase molecule, glycerol often binds to the enzyme in powder form and hereby makes the access of the hydrophobic oil molecules difficult (Kristensen *et al.,* 2005; Yang *et al.,* 2005a; Bellot *et al*., 2001). The use of suspended enzyme povders tends furthermore to aggregate and attach to the wall of the reactor (Barros *et al.,* 1998). This leads to mass transfer limitations with inefficient reactions (Ison, 1994; Murty *et al.,* 2005). Thus it is necessesary to stabilize and/or fix the enzyme in the reactor to make a large proportion of the active site accessible for both substrates and to make the lipase robust under physical handling. Hence, it is not possible to 'adopth' the process principle from chemical glycerolysis to enzymatic glycerolysis as it is. 'Adjustments' is required to make it suitable for enzymatic glycerolysis by introducing 'alternative' techniques.

A literature and patent survey shows that many techniques have been tested to overcome some of the 'lipase' and 'low temperature' induced challenges in enzymatic glycerolysis (Table 5.1 and Table 5.2). Since the enzymatic glycerolysis ends with a MAG and DAG mixture the strategy can be different depending on the desirable product formation. The solvent free glycerolysis system leads for instance in general to a high DAG formation but only a limited MAG formation in reasonable time (Table 5.1). With a present objective to obtain a system that favors MAG rather than DAG the involvements of alternative techniques or the use of assistance media is believed necessary to achieve suffient high MAG yields (Table 5.1).

Table 5.1: An overview of major literature published about lipase-catalyzed glycerolysis process for production of MAGs the last decade

Substrates:		**Oil:gly ratio**	**Temp (°C)/ Time**	**Reaction system**	**Product composition: % w/w**			**Lipase**	**References**
Acyl donor	**Acyl acceptor**				**MAG**	**DAG**	**TAG**		
Sunflower Anchovy	Glycerol	1:2 mol/mol	40°C/ 6 h	Solvent free		53 47		Lipase D "Amano" 100 *Rhizopus delemar* , 1,3-specific	Tüter *et al.,* 1998
Palm oil Palm kernel oil	Glycerol	1:2 mol/mol	40°C/ 24 h	Solvent free		66 64		Lipase D "Amano" 100 *Rhizopus delemar* , 1,3-specific	Tüter *et al.,* 1999
Palm oil Palm kernel oil	Glycerol	1:2 mol/mol	40°C/ 24 h	Solvent free	18 31	38 42	23 18	Commercial SP 398 *Humicola langinosa*, 1,3-specific	Tüter & Aksoy, 2000
Tallow	Glycerol	1:2 mol/mol	50°C/24 h	Solvent free Solid phase	35			Immobilzed *Mucor meihei* Lipozyme	Stevenson *et al.,* 1993
Crude vinyl laurate	Glycerol	1:5	55/25°C/ 96 h	Solvent system: n-pentane	5	38	57	Non-specific *Pseudonomas cepacia*	Bornscheuer *et al.* , 1994
Crude vinyl laurate	Glycerol	1:5	55/25°C/ 96 h	Solvent free system	13	82	5	Non-specific *Pseudonomas cepacia*	Bornscheuer *et al.* , 1994
Crude trilaurin	Glycerol	1:3	55/25°C/ 96 h	Solvent free Solidification	96	2	2	Non-specific *Pseudonomas cepacia*	Bornscheuer *et al.* , 1994
Triolein	Glycerol		25°C (8 h)/8°C 60 h	Solid phase system				Imomobilzed *Pseudomonas cepacia* on Celite	Bornscheuer *et al.* , 1994
Butter oil	Glycerol	3:2	35-40°C/ 8 h	Solvent system: t-butanol	22	25	51	Immobilized in gel	Yang & Parkin, 1994

Substrates:		Oil:gly ratio	Temp (°C)/ Time	Reaction system	Product composition: % w/w			Lipase	References
Acyl donor	Acyl acceptor				MAG	DAG	TAG		
Olive oil	Glycerol	1:3	80°C/4 h	Solvent-free	25	50	30	Immobilzed lipase	Coteron *et al*., 1998
Olive oil	Glycerol		37°C/ 14 h	Reverse micelles Solvent: isooctane			80	Immobilized *Chromobacterium viscosum*	Chang *et al*., 1991
Olive oil	Glycerol	1: 4.8	25°C/ 1 h/ 10°C 10 h/ 5°C 61 h	Solid phase reaction	90			*Pseudomonas sp.* KWI-56 Lipase (PSL)	Rosu *et al.,* 1997
Mink whale oil	Glycerol		12/5°C/21 days	Solvent free	53	25	19	Lipase Ak from *Pseudomanas spp.*	Myrnes *et al*. 1995
Beef tallow	Glycerol	2:3/1:2.5	38-46°C	Solid phase system	70			*Pseudomonas fluorescens Chromobacterium viscoosum*	McNeill *et al.,* 1990 McNeill & Yamane, 1991
Olive-pomace oil	Glycerol		40-60°C	Molecular sieve to adsorb produced water				*Candida Antarctica* lipase B -Novozym 435	Fadiloglu *et al*., 2003
Triolein	Glycerol		40°C/5 h	Silica adsorbed glycerol Hexane/2-methyl-2-butanol system	60	25	15	*Rhizomucor mieheii (Lipozyme IM-20)*	Rendon *et al.,* 2001
Pure TAG Palm oil	Glycerol		40°C/ 24 h	Solvent free	52	26	14	*Pseudomonas fluorescens* (PS)	McNeill & Berger, 1993

Substrates:		Oil:gly ratio	Temp (°C)/ Time	Reaction system	Product composition: % w/w			Lipase	References
Acyl donor	**Acyl acceptor**				**MAG**	**DAG**	**TAG**		
Soybean Oil	Glycerol	1:7	60-70°	Solvent system: Supercritical Carbon Dioxide	Up to 87%			Novozym 435	Jackson & King, 1997
Palm olein	Glycerol	1:8	45°C/24 h	Acetone/isooctane 3:1 v/v	56%			Lipase PS (*Pseudomonas)* immobilized onto Accurel EP100	Kaewthong & H-Kittikun, 2004
Palm Oil		1:2.7	45°C/24 h 45°C	Batch PBR	28% 14%			Lipase PS (*Pseudomonas)* immobilized onto Accurel EP100	Kaewthong *et al.,* 2005
Butter oil	Glycerol	2:1	45-55°C/10 h	continuous reactor Solvent free	24%	47%	30%	*Pseudomonas cepacia*	Garcia *et al*., 1996
Olive oil	Glycerol	1:9	30°C/27 h	n-hexane system	50%			Candida rugosa lipase (Lipase AY from Amano) immobilized onto polyurethane foam	Ferreira-Dias & daFoncesa, 1995
Soybean Oil	Glycerol	1:2	40°C/ 1h		25mol %			Lipses from *Pseudomonas sp.*	Noureddini & Harmeier, 1998
Sunflower oil	Glycerol	1:4.5	40°C/30 min	Tert-butanol PBR-system	70%			*Candida Antarctica* lipase B -Novozym 435	Yang *et al.,* 2005a + 2005b
Campher tree seed oil/cocoa-butter	Glycerol	1:2.7	25°/7°C/7 hours	Solid-phase system	90%			*Chromobacterium viscosum* lipase	Thude *et al.,* 1997

Table 5.2: Patents involving methods for producing MAG by lipase catalyzed reactions.

Applicant	Patent ID	Inventor	Title	Description
Nippon Suisan Kaisha Ltd. (Tokyo, Japan)	WO 2/006505 EP1300470A1	Irimescu *et al.,* 2003.	Process for the production of glycerides with lipases	Alcoholysis of starting TAG with EPA C20:5n-3)/DHA (C22:6n:3) long chain fatty acids with one 1,3-specific lipase that can act even on long-chain fatty acids (Novozym®435) to form 2-MAG. Ethanol was present in the system and acetone was added as solvent. Producing TAG of 2-MAG with fatty acid ester (ethyl octanoate) or free fatty acid (octanoic acid=caprylic acid C8, decanoic acid, capric acid C10) with another 1,3-specific lipase (Lipozyme IM). Water was present in the system
Maruha Corp. (Osaka City, Japan	JP8214892	Tominaga *et al.,* 1996.	Production of partial glyceride containing highly unsaturated fatty acid	Oil/or fat (tuna oil) and glycerol is reacted with a lipase such as *Pseudomonas fluorescens* at 30°C to achieve partial glycerides containing highly unsaturated fatty acids.
Opta Food Ingredients Inc. (U.S.)	US5316927	Zaks & Gross, 1994.	Production of monoglycerides by enzymatic transesterification	Oil or pure TAG are combined with alcohol, a small amount of water and a lipase to produce high yields of β-MAG (2-MAG). Expired 2002 due to failure to pay maintenance fee
European Economic Community (Luxembourg)	EP0445692A3 JP3262492	McNeill *et al.,* 1991.	A method of producing monoglyceride	Glycerolysis reaction between fat or oil and glycerol using a lipase enzyme as catalyst. Reaction carried out in a liquid-liquid emulsion state and afterwards in a slurry solid state.
Wuxi Light Industry university (China)	CN1244587	Zhang *et al.,* 2000.	Technological process of utilizing lipase in solid phase catalytic glycerination of oil and grease	Reaction between glycerine, oil, enzyme and water in reactor with controlled warm water bath, thermometer, and mechanical stirrer. Achieve MAG content of 40-78%.

Applicant	Publication no.	Inventor	Title	Description
Kao Corporation (Tokyo, Japan)	EP1111064A1 US6361980 JP2001169795	Sugiura *et al.,* 2001.	Process for the enzymatic preparation of diglycerides	Esterification reaction between a fatty acid/lower alcohol/ ester and glycerol/MAG in an enzyme –packed tower with immobilized enzyme preparation (Lipozyme IM). Repeated reaction under a high pressure lead to the risk for compacting the enzume increasing the pressure drop. Unreacted glycerol, lower alcohols, MAGs are removed by conventionally well-known processes such as molecular distillation
Maruha Corp and Watanabe Yoshi (Japan)	JP2004168985	Shimada *et al.,* 2004.	Omega-3 type highly unsaturated fatty acid-containing partial glyceride composition and its production	Method for efficiently producing partial glycerides with ώ-3 type highly unsaturated fatty acids, especially MAG. Esterification reaction between PUFA or ester and glycerol under reduced pressure atmosphere in the presence of a lipase which only recognize MAG and DAG as substrate and not TAG.
Enzytech Inc. (U.S.)	WO/90/13656	Zaks & Gross, 1990.	Enzymatic production of glycerides containing omega-3 fatty acids	Lipase catalyzed transesterification of TAG fish oil in alcohol medium containing some water. MAGs are then separated by dissolving the MAGs enriched in omega-3 fatty acids in organic solvent, which causes selective low temperature crystallization of the MAGs with predominant saturated profile. The solvent containing the omega-3 enriched MAGs is evaporated, leading to a highly pure MAG product.
Rinoru Oil Mills Co. Ltd. (Osaka City, Japan)	JP2003113396 US2003/0130533 A1	Yamauchi *et al.,* 2003.	Conjugated Fatty Acid containing monoglycerides and process for producing them.	Monoglyceride preparation by estrification between free fatty acids containing a conjugated fatty acid and glycerol using lipases as catalyst at 0-20°C Or, a one reaction system with continuous esterification and glycerolysis. To progress the glycerolysis after esterification, the reaction mixture is solidified by cooling

Solid phase crystallization of MAGs is beneficial to drive the reaction towards higher MAG yields by continuously isolate and remove produced MAG (Bornscheuer *et al.,* 1994; McNeill *et al.,* 1991; Rosu *et al.,* 1997; Xu, 2003). Glycerol adsorbed to silica gel is advantageous to obtain a widespread distribution of the glycerol and to make the lipase more accessible for the lipid substrate (Bellot *et al.,* 2001; Bornscheuer, 1995). The usage of protected glycerol is attractive to achieve specific sn-positioned MAGs and avoid acyl-migration (Bornscheuer, 1995; Akoh, 1993). Encapsulation of glycerol into reverse micelle is attractive to overcome the low solubility of glycerol by enhanced interfacial area (Bornscheuer, 1995). The use of lipase in an immobilized form is beneficial to enhance the enzyme stability and to make it widely distributed into a greater surface area (Elfmann-Börjesson & Härröd, 1999). Water addition is beneficial to enhance the lipase activity owing to its water/lipid interfacial activation (Yang *et al.,* 2005a; Cheirsilp *et al*., 2007). Reaction performed in different medias like supercritical CO_2 (Jackson & King, 1997) or organic solvents (Bornscheuer *et al.,* 1994; Kwon *et al*., 1995; Rendon *et al.,* 2001; Yang *et al*., 2005a) are tested beneficial for improved reaction efficiency by improved reactant miscibility.

Although many strategies/technologies are available for MAG production through enzymatic glycerolysis only few of these approaches are believed suited for industrial utilization. The patent survey demonstrates an interest for utilizing the alcoholysis reaction and the solid phase crystallization technique commercially (Table 5.2). Even so this approach is not believed optimal for developing a continuous, easy operated, efficient and cost effective process. MAG solidification leads to impractical batch wise handling although it can be conducted in a second separator vessel (Aha *et al.,* 1998). In most patented alcoholysis processes (Irimescu *et al.,* 2003; McNeill *et al.,* 1991; Zhang *et al.,* 2000; Tominaga *et al.,* 1996) water is present which leads to unwanted FFA formation due to hydrolysis reactions between the water and oil. From a commercial point of view silica gel and assistance of organic media takes up costly space in the reactor and complicates the subsequent purification. Protected glycerol and reverse micelle systems requires expensive extra processing or costly raw material investments. Immobilized lipase catalysts are in general costly (Kaewthong *et al.,* 2005). Hence the selected strategy must be a

compromise between the expected benefits and drawbacks. A recent study of enzymatic glycerolysis conducted in an enzyme packed bed reactor (PBR) with the assistance of a TB medium enhanced the reaction efficiency in such a way that it seems very promising for future industrial applications. Hence, from applied point of view, the use of assistance media and immobilized enzyme PBR is considered as very potential approaches to commercialize the glycerolysis process on an industrial scale (Yang *et al*., 2005a; Goto *et. al.,* 2005; Garcia *et al.,* 2001, Bellot *et al.,* 2001; Murty *et al*., 2005; Moreno *et al.,* 2005).

5.1 Solvent engineering

Since Zaks and Klibanov discovered that lipases are active in non aquous environments (Zaks & Klibanov, 1984; Zaks & Klibanov, 1985) solvent engineering of lipase catalyzed reactions in micro aqueous media has been subject to various studies. This is due to greatly enhanced technological utilization of the lipases by switching from their natural aqueous reaction media into organic solvent media (Klibanov, 2001). The solvent lipase-lipid system is a surfactant modificated system with a water-limited environment. Here, the enzymes are coated with a hydration layer of water, essential for catalytic activity and solubilized in a bulk organic phase. The water amount is just sufficient to hydrate the enzyme and allow it to stay locked in an active form while the solvent do not remove the essential water layer (Zaks, 1991; Klibanov, 2001; Paez *et al.,* 2003).

The benefits of using solvent are to enhance the lipase stability and/or the activation which leads to improved reaction efficiency (Graber *et al,* 2007; Grochulski *et al.,* 1993; Klibanov, 2001). The active lipase site can interact with the solvent leading to change in enzyme conformation and/or flexibility. In CALB the active site is for instance accessible to external solvent through a narrow channel in which the walls are very hydrophobic. This solvent accessible site suggests that the enzyme can adopt a conformation close to an activated state when solvent is employed (Uppenberg, 1994). This solvent induced lipase ‘activation’ can explain the enhanced efficiency in some organic media. Solvents also helps with dissolvation of hydrophobic and/or immisible substrates. By that a fluid solvent system is formed

with better partitioning/distribution between the active site of the enzyme and the medium. Owing to insolubility of the hydrophilic lipase protein structure and water in hydrophobic organic media less of the essential tightly lipase bounded water, is also stripped of (Graber *et al,* 2007; Klibanov, 2001; Grochulski et al, 1993; Zaks, 1991; Martinelle & Hult, 1995; Uppenberg, 1994).

The solubility effect of the solvent in lipid systems is often expressed by the octanol-water partition coefficient (P) (Monteiro *et al.*, 2003; Graber *et al.,* 2007; Rendon *et al.,* 2001; Lee *et al.,* 2004; John & Abraham, 1991). In general, P (or, in its more common form of expression, log p) provides a measure of the lipophilic versus hydrophilic nature of a compound. P describes the distribution of a compound in a two-phase system. It is defined as the ratio of the equilibrium concentration of the compound in a 1-octanol-rich phase (hydrophobic) to the concentration in a water-rich phase (hydrophilic) in which water and 1-octanol are in equilibrium (Lide, 2007a). Log p tends to be largest for compounds with extended non-polar structures and smallest or negative for compounds with highly polar groups. In the glycerolysis sytem, glycerol contribute with a log p value of -1.76 and long chained PUFAs from TAG oil such as linoleic acid (C18:2) and oleic acid (C18:1) contribute with log p values of ~7.1 and ~7.6, respectively (Lide, 2007a). Lipases have shown good stability and activity in hydrophobic solvents with $2 \leq \log p \leq 4$, like *n*-hexane (nH), but in such non-polar media is the glycerol insoluble. In an esterification reaction between glycerol and FFA the more polar solvents, including tertiary alcohols with $\log p \leq 2$, provides a more homogenous system that favoured the MAG synthesis (Monteiro *et al.,* 2003).

Even though applying solvent to lipase catalyzed reactions is believed greatly beneficial for enhanced reaction efficiency it also enhances the risk for lipase inhibition. Owing to the plausible solvent-lipase interactions the solvent can act as competitive substrate to the reactants (Graber *et al.,* 2007). Hence, it is important to make a good choice of solvent that function as an inert carrier material for the reactants to the active lipase site during reaction. In lipase-catalyzed interesterifications reactions solvents such as dioxane, nH, *n*-heptane, acetonitrile, acetone, isooctane, TB and *tert*-pentanol (TP) have successfully been used (Bellot *et*

al., 2001; Elfmann-Borjesön & Härröd, 1999; Goto *et al.,* 1995; Hess *et al.,* 1995; Kaewthong & H-kittikun, 2004; Rendon *et al*., 2001; Yang *et al.,* 2005a; Lee *et al.,* 2004). These solvents cover varied polarities and chemical structures and are mixed in different ways. Their effect very much dependent on the set up of the reaction system such as reactants included, FA composition and the specific type of lipase used. For instance, the *Aspegillus terreus* lipase exhibited high estrerification degree between different types of organic alcohols media and stearic acid (C18:0), in contrast to the monounsaturated oleic acid (C18:1), which did not (Gulati *et* al, 2003). In alcoholysis reactions between various alcohols and TAG hexane was needed as co-solvent to enhance short chain MAG synthesis (Lee *et al.,* 2004). Ketones and tertiary-alcohol inhibited the CALB-lipase in transacylation reaction between methyl propanoate and 1-propanol (Graber *et al.,* 2007). Contrary to that the tertiary alcohol TB enhanced the glycerolysis reaction between oil and glycerol dramatically (Yang *et al.,* 2005a). Owing to these varied effects, it is difficult to establish general rules that can help to find the optimal solvent. However, in glycerolysis, organic alcohol media compete in general with the glycerol in the following order: Primary alcohols ≥ secondary alcohols ≥ tertiary alcohols (Peng *et al.,* 2000). Hence, the primary and secondary alcohols solvent media are believed unsuitable. In contrast, the tertiary alcohol TB seemed suitable ascribed a sterically hindered tertiary hydroxyl group (–OH) towards the CALB-lipase in the specific set up (Yang *et al.*¸2005a).

5.2 Immobilized lipases

A broadly used strategy in lipase catalyzed reactions is to employ the lipase in an immobilized form (Bellot *et al.,* 2001; Plou *et al.,* 1996; Elfmann-Borjesön & Härröd, 1999; Goto *et al.,* 2005; Kaewthong *et al.,* 2005; Barros *et al.,* 1998; Nakaoki *et al.,* 2005; Xu, 2000). Here, the enzyme is chemically or physically attached to a solid 'carrier' or 'support material' or entrapped in matrices through which the substrate can diffuse. The principle of the glycerolysis reaction with immobilized lipase is illustrated in Fig 5.2.

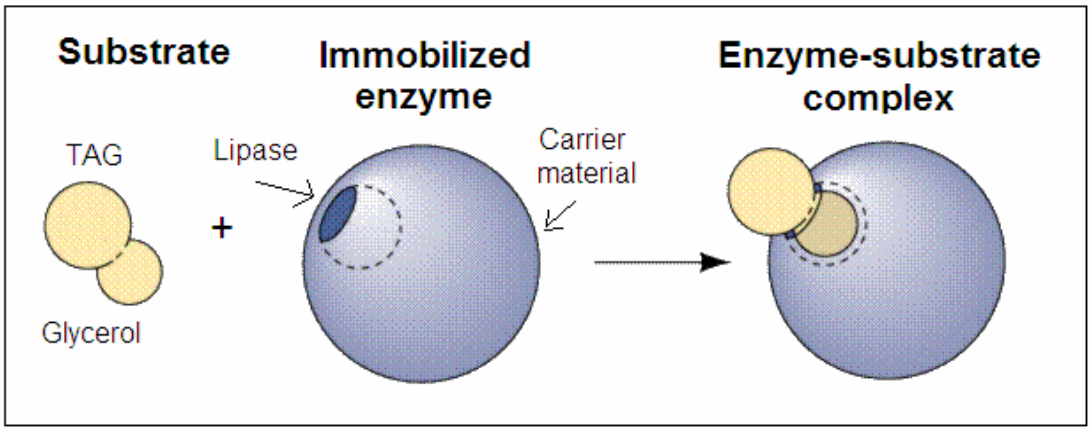


Figure 5.2: Illustration of the substrate binding to an immobilized enzyme

The benefits of using lipase in an immobilized form are various. By 'fixing' the enzyme to a carrier the enzyme is spread on a large area. By that formation of aggregates is avoided, a larger proportion of the active sites becomes available for catalytic function and the mass transfer of reactant and product is facilitated. The large and solid immobilized lipase complexes are easy separated from batch operations and allow reutilization. It is also easier to pack immobilized enzyme into a column for continuous processing and the immobilization often has a stabilizing effect with extended long term use at elevated temperatures (Barros *et al.,* 1998; Nakaoki *et al.,* 2005). For instance, esterification reactions of lauric acid in isooctane system has shown an 51-fold increase in activity when the lipase was employed in immobilized form compared to in its native form (Goto *et al.,* 2005).

For the use of immobilized enzymes the structure and size of the carrier material and the location and amount of the attached lipase are of great importance for the reaction performance. The enzyme located in a layer adjacent to the surface of the carrier material is beneficial to reduce the effects of 'internal mass transfer' limitations. Internal mass transfer covers the substrate diffusion inside the porous catalytic particle (Ison *et al.,* 1994; Murty *et al.,* 2004). On the other hand, a high lipase loading on the surface enhances the risk for reduced activity owing to a tightly packed lipase monolayer which can make some of the active sites inaccessible to the substrate (Ison *et al.,* 1994). A large available surface area of the immobilized catalyst can increase the contact between the substrate and the active lipase site with enhanced maximum reaction rate. This can be obtained by reducing the size of the catalyst particles which provides a greater surface area per unit catalyst volume or by using

porous support materials. However, by using a porous carrier the diffusion of the substrate through the porous matrix into the active site of the enzyme has the risk of internal mass transfer limitations. Also small particles increase the magnitude of any pressure drop. Regular–shaped catalyst particles are much better at reducing high pressure drops and irregular flow patterns (Ison *et al.,* 1994; Xu *et al.,* 1998; Nakaoki *et al.,* 2005; Murty *et al.,* 2004). The stability of the support material in organic media is also of major importance. Swelling of carriers in organic media is for instance seen by the poly methyl methacrylate (PMMA) material used in Novozym®435 (Kaneda & Vincent, 2004; Ruzzu & Mathiss, 2002).

Many support materials such as calcium carbonat ($CaCO_3$), silica gel, Celite (also known as kieselguhr), ion-exchange resins (such as the macroporous anion-exchange resin Duolite), polypropylene (Accurel) and macropourous acrylic resin have been used for immobilization of lipases (Kaewthong *et al.,* 2005; Kristensen *et al.,* 2005). Hydrophobic or neutral carriers have been shown to be most suitable for high catalytic efficiency in glycerolysis and esterification reactions of lipids (Kristensen et al, 2005; Goto *et al.,* 2005). In the glycerolysis system especially *Candida antractica* lipase B physically immobilized to the hydrophobic (PMMA) has previously been demonstrated superior to have a unique qualification (Yang *et al.,* 2005a; Kristensen *et al*, 2005). Hydrophilic carriers are in general found inappropite for the glycerolysis reaction owing to the tendency to adhere to the glycerol. Hereby, the contact to the oil phase is restricted and a properly substrate dispersion is hindered (Kristensen *et al.*, 2005).

5.3 Enzyme packed bed reactors

The use of PBR is a common strategy to facilitate lipase-catalyzed glycerolysis and other fat interesterification reaction in an efficient and relatively simple way. Several investigations have confirmed the benefits of PBR with immobilized lipase catalysts for lipid modification (Murty *et al.,* 2004; Ison *et al.*, 1994; Garcia *et al*., 1996; Xu *et al.,* 2002b; Yang *et al.,* 2005a; Watananbe *et al.,* 2005; Arcos *et al.,* 2000; Moreno *et al.,* 2005). The PBR is characterized by a high density loading of the immobilized enzyme and a ‘fixed enzyme bed’ in which the

substrate passes through. This promotes the enzyme contact to the reactant mixture and makes long term production as well as continuous and simple product separation from the catalyst feasible. Hence such a set up is suitable for commercial utilization and helps to overcome the in general high immobilized enzyme costs (Kaewthong *et al.,* 2005; Moreno *et al.,* 2005).

In the PBR, the time in which the substrate is in contact with the enzyme can be calculated by a 'residence time' which is based on the enzyme bed volume, substrate flow rate and void fraction, the latter calculated as the ratio between the substrate- and the enzyme bed- volume in the reactor (Xu *et al.,* 1998). However, when applying the enzyme at varied weight loadings a weight based reaction time can be a useful alternative to eaily implement data into different set ups without considering the changing void fraction. This weight based reaction time covering plug flow reactors containing catalyst particles is defined in equation 5.1 (Levenspiel, 1999):

$$\tau = \frac{W}{\nu_0 \cdot \rho} \qquad (5.1)$$

τ = reaction time (min)
W = weight of catalyst pellets on dry matter basis (g)
ρ = bulk density of the dry enzyme (g/cm^3)
ν_0 = volumetric flow rate of the reactant mixture (cm^3/min)

An optimal and consistent movement of the fluid to the immobilized enzyme bed, through the catalyst pores and away from the enzyme bed are of great importance for efficient PBR performance and and operational stability. These phenomena can be exploited by 'mass transfer' investigations of factors affecting it such as the reactant flow rate, the catalyst design, and column dimension. Mass transfer effects in differnt fat interesterification reactions have been performed although the numbers of investigations are very limited for the glycerolysis reaction (Murty *et al.,* 2005; Ison *et al.*, 1994; Garcia *et al.*, 1996; Xu *et al.,* 1998; Yang *et al.,* 2005b; Murty *et al.,* 2004). External mass transfer covers the transport (flow) between the bulk solution and the outer surface of the enzyme particle. External mass transfer limitations are in generally reduced in PBR by increased flow rate of the substrate through the column or reduced reactor height-to diameter ratio, eventually leading to a higher linear velocity (Ison *et al.,* 1994). By that the ability of the reactant mixture to penetrate the

support structure of the immobilized enzyme bed becomes more easily. However increased flow rate enhances the risk of pressure drops wich complicates the operation of the bed. The pressure drop can be further facilitated by long term operations, 'long' enzyme beds and down stream flow, typically used for industrial scale operations owing to the gravity forces. These factors potentially have a compressing impact on the enzyme bed with risk of conformational changes (Zhang, 2004; Xu *et al.,* 1998).

To characterize/define the flow behavior through the the column calculations of Reynolds number (Re) can be performed (Lide, 2007c):

$$\text{Re} = \frac{d_p \cdot \upsilon \cdot \rho}{\mu} \qquad (5.2)$$

d_p is the enzyme particle diameter (mm)
υ is the fluid velocity (flow/cross area of column)
ρ is the fluid density (kg/ m^3)
μ is the fluid viscosity of the reactant mixture (mPa · s)

The flow can hereafter be characterized as turbulent if Re ≥ 4000 which tends to have a fluctuating behavior or laminar if Re ≤ 2000 characterized by smooth and constant fluid motion (Lide, 2007c).

The Sherwood number (Sh), sometimes termed the mass transfer Nusselt number, can be used to predict the fluid moving past single particles in external mass-transfer investigations. It calculates the ratio between convective and diffusive mass transport (controlled and random movement, respectively), by following equation (Levenspiel, 1999):

$$\text{Sh} = \frac{k \cdot d_p}{D} = 2 + 0.6\,(\text{Re})^{1/2}\,(\text{Sc})^{1/3} \qquad (5.3)$$

k= mass transfer coefficient
d_p = enzyme particle diameter (mm)
D = molecular diffusion coefficient (m^2/s)
Sc = Schmidt number = $\mu/ \rho \cdot D$
μ = fluid viscosity of the reactant mixture (mPa · s)
ρ = bulk density of the dry enzyme (g/cm^3)

5.4 Membrane technology

In the last few years there has been an increasing interest in the application of membrane technology to separate lipids by semipermeable ultrafiltration (UF) mebranes. The use of membranes is a promising continuously operated alternative to more energy consuming separation techniques requirering high temperatures. The major applications of membrane technology in the oil and fat technology is in the recovery of solvent from micelle, separation in degumming, refining and bleaching, catalyst recovery and hydrolysis of oils and fats or glyceride synthesis. Most of these industrial applications are related to aqueous solution. (Vikbjerg *et al.,* 2006; deMoura *et al.,* 2007; Manjula & Subramanian, 2006; Kumar & Bhowmick, 1996; Xu *et al.,* 2000). However, there is an enormous potential field for applications in non-aqueous system, provided that membranes suitable to organic solvent are available (Vikbjerg *et al.,* 2006). Also in MAG processing by the glycerolysis reaction UF is considered as a very potential separation technique to purify MAGs and other 'high weight' lipid molecules from the low weight glycerol and solvent.

Porous UF membranes can force low molecular weight liquid to pass through a semipermeable membrane by pressure while high molecular weight lipids are retained. The size and shape of the components relative to the pore size in the membrane determine the separation. Molecular weight cut-off (MWCO) value of 1,000 and 10,000 are commonly used values for oil/lipid refining/purification by membrane technology (Manjula & Subramani, 2006; Vikbjerg *et al*, 2006). Membranes are typically pretreated or conditioned in organic solvent to wash out undesirable by-products from the manufacturing process (deMoura *et al.,* 2007). Even so solvents typically have a swelling effect on porous membrane material, thereby changing the pore sizes and the solvent permeability at long time use. Hence, the polymer solubility in different solvents is of great importance and can be determined by the use of Hansens parameters. By such calculations a support layers prepared from polysulphone (PSf) or a polyethyleneterephthalate (PET) was found to be most solvent resistant in a in hexane and caprylic acid system (Vikbjerg *et al.,* 2006). Also membranes made of polyvinylidenefluoride (PVDF) seem to be among successful materials to avoid deformations in some solvents such as hexane and methanol (Vikbjerg *et al.,* 2006; Manjula & Subramanian, 2006).

EXPERIMENTAL AND ANALYTICAL WORK

6. Experimental approaches and methodologies

A brief description and some of the thoughts for choosing the selected experiments are given for each of the main areas of experimental work conducted. For more detailed descriptions and illustrations please refer to the corresponding Papers or Appendices.

6.1 Analytical approaches

6.1.1 TLC-FID methodology

The Iatroscan MK 6 apparatus and the ancillary equipment, all supplied by SES GmbH, Bechenheim, Germany were used for determination of MAG, DAG and TAG content from glycerolysis samples by the principle of thin layer chromatography –flame ion detection (TLC-FID). This analytical method was chosen due to its accessibility, simplicity, rapidity and the general popularity as separation techniques in the fields of lipids, oils and fats (Peyrou *et al.*, 1996; Striby *et al.*, 1999; Nishiba *et al.,* 2000; Rosas-Romero *et al.,* 1996; Shanta & Napolitano, 1998).

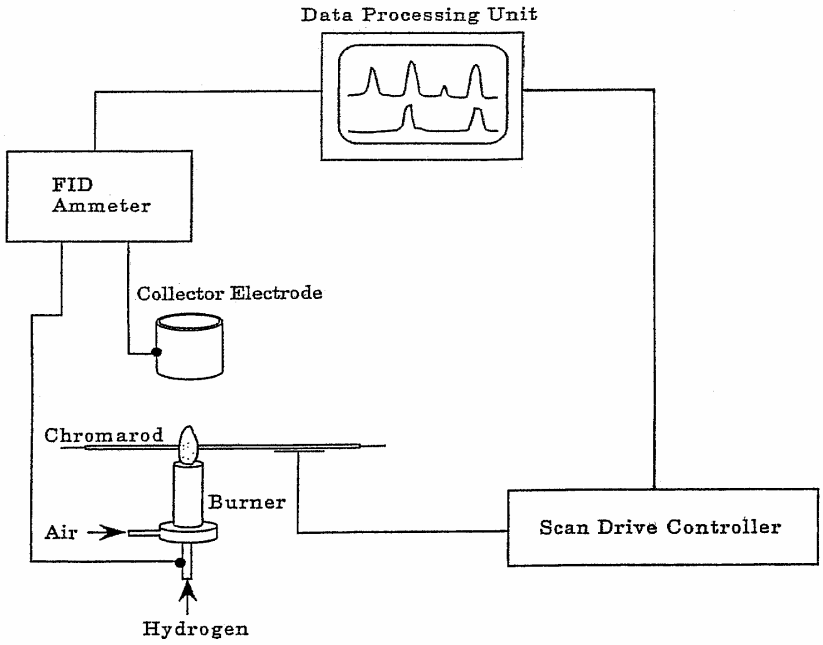


Figure 6.1: Principle of the TLC-FID methodology. Adapted from Iatroscan, 2001.

By the TLC-FID methodology (Fig. 6.1) sample components (spotted onto Chromrods) were separated by a solvent mixture that forced the acylglyceols to

migrate according to polarity. The rod traversed hereafter through a hydrogen flame to ionize the sample. Due to the high voltage on the FID electrodes, minus-ions moved to the burner and plus-ions to the collector electrode, which generated an ion flow between the burner and collector proportional to the mass of the component. This ion signal was then converted to mV and send to a data processing unit providing the obtained chromatograms (Iatroscan, 2001).

Varied solvent compositions, based on a literature screening, were tested to obtain a clear and sharp separation of the acylglycerols on the Chromarods in a single step development (Peyrou *et al.*, 1996; Striby *et al.*, 1999; Nishiba *et al.,* 2000; Rosas-Romero *et al.,* 1996; Shanta & Napolitano, 1998; Freedman *et al.,* 1984; Iatroscan, 2001). The selected solvent mixtures are summarized in Table 6.1.

Table 6.1: Screened solvent mixtures for sample separation on TLC-FID rods.

ID-number	Solvent mixture	Ratio (ml)
A	chloroform: acetone	63:7
B	chloroform: acetic acid	70:1
C	chloroform: acetonitrile:acetic acid	60:10:1
D	chloroform: hexane:acetic acid	55:15:1
E	chloroform: hexane:acetic acid	60:10:1
F	hexane: ethylether:acetic acid	55:15:1
G	heptane:2-propanol:acetic acid	63:7:1
H	n-heptane: diethylether:acetic acid	35:35:1
I	dichlormethane:hexane:acetic acid	50:20:1
J	dichlormethane:hexane:acetic acid	60:10:1
K	dichlormethane:hexane:acetic acid	40:30:1

The suitability of pure standards as well as internal standard (IS) for MAG, DAG and TAG quantification were tested. Standard curves were made from pure MAG, DAG and TAG components. IS were used to calculate a response factors (R_f) and from here the amount of each component (%X) by Eq. 6.1 and 6.2:

$$R_f = A_{IS} \cdot W_X \; / \; A_X \cdot W_{IS} \qquad (6.1)$$

$$\%X = A_X \cdot R_f \cdot W_{IS} \cdot 100 \; / \; A_{IS} \cdot W_S \qquad (6.2)$$

A_{IS} = Measured peak area of IS
W_{IS} = Weight of IS
A_X = Measured peak area of component X
W_X = Weight of component X
W_S = Weight of sample

The different standards used are summarized in Table 6.2.

Table 6.2: Standards used in the optimization of the TLC-FID method.

Standard	Composition		Obtained from
Mono-olein, Di-olein (9c) Tri-olein (9c)	minimum 99% pure		Larodan Fine Chemicals, Malmö, Sweden.
Internal std. 2	46.4 wt% $MAG_{total,}$ 41.1 wt% DAG_{total} and 11.5 wt% TAG_{total}	Predominant fatty acids: C14: 3.9 wt% C16: 30.0 wt% C18: 61.0 wt% Other: 5.1 wt%	Danisco A/S, Brabrand, Denmark.
Propylgallate	minimum 99% pure		VWR Internal Ltd., Albertslund, Denmark.

The accuracy of the method was clarified by four repeated measurements of the internal std. 2 at varied concentrations. The TLC-FID measurements were compared to similar GC-FID measurements on identical samples after glycerolysis reaction in a TB system. Further details about the TLC-FID optimization work, elaborated descriptions of the TLC-FID procedures and a user manual for operating the Iatroscan MK-6 are found in Appendix I. In Paper I is the optimized TLC-FID method described.

6.1.2 GC-FID method

The optimized TLC-FID method showed some analytical limitations on the actual glycerolysis samples arising from the lack of glycerol identification and uncertain compound quantification. These limitations pointed towards implementation of a supplementary analytical technique to provide the required detailed picture of the glycerolysis process in certain parameter studies. A capillary gas chromatography (GC) -FID analytical technique, currently used by Danisco A/S, was considered to be a well suited alternative. Danisco A/S utilizes this established method for analysis of MAG and other emulsifiers. It is a highly informative method capable of separating and quantifying all major and minor products from the chemical catalyzed glycerolysis reaction in approximately 30 minutes (Danisco A/S, 1989; AOCS, 1997). Due to similarities in product composition from chemical and enzymatic glycerolysis it could be applied to present samples without further optimization. Thus, over time the capillary GC-FID method was adopted from Danisco A/S.

The capillary GC-FID method was based on the principle of the AOCS Official Method Cd 11b-91 (AOCS, 1997). It included initially silyation of the free hydroxyl groups (-OH) to stabilize the sample and protect against acyl-migration followed by high temperature capillary GC above 300°C and the principle of FID-detection. Components were quantified as the weight% in the sample determined by the use of R_f (from IS) and areas under the curve for each peak (in the obtained chromatogram) (Danisco Cultor, 2000; Danisco Ingredients 1998/99). In Paper I is the used GC-FID method more detailed described.

6.1.2 Statistical data analysis

In most cases, a conventional statistical approach was used, where one variable was varied at a time while all other variables were kept constant. The Excel Analysis Toolpack: Analysis of Variances (ANOVA): Single Factor was used to test for significant differences established at $P \leq 0.05$. This 'simple' approach was selected since a great part of the experimental work was on the initial screening phase with more comprehensive statistical analyses believed not to be required.

A more careful multivariate dataanalysis by the response surface methodology (RSM) was however found useful for optimizing the glycerolysis system in the most suited single solvent system. The RSM was found appropriate owing to several parameters defined in a sufficient 'narrow' range. The benefits from using RSM compared to single experimental design were a reduced numbers of experiments while the same amount of information was obtained. Furthermore interactions between the tested factors were included in the data analysis owing to different mathematic and statistical models included (Xu, 2002c; Montgomery, 1997). The software Moddde 7.0 from Umetri (Umerå, Sweden) was used to analyse for main effects and interactions from a three-level four-factor experiment.

6.2 MAG + DAG synthesis by enzymatic glycerolysis

The glycerolysis reaction route was selected to take advantages of the extensively studies in this field and to utilize the relatively cheap and easy accessible

vegetable oils as lipid raw materials. In addition, by using the glycerolysis reaction routes, the project benefit from the industrial know how related to current applied chemical glycerolysis that feature similarities to the enzymatic glycerolysis. Rape seed oil and sunflower oil was selected as neutral flavoured TAG raw material carrying nutritional important PUFAs. Both rape seed oil and sunflower oil are rich in monounsaturated oleic acid: C18:1 (n-9), and polyunsaturated linoleic acid: C18:2 (n-6) or linolenic acid: C18:3 (n-3) as illustrated in Table 6.3.

Table 6.3: Fatty acid composition of some of the oil used.

Fatty acid	Rape seed oil	Sunflower oil
C15≤	0.3	0.1
C16	4.9	6.7
C16:1	0.3	0.2
C17	0.2	0.1
C18	1.8	3.7
C18:1	60.3	26.3
C18:2	20.7	61.2
C18:3	8.7	0.4
C20 ≥	2.8	1.3
Sum	100	100

Sunflower oil was provided from Aarhus United, Aarhus, Denmark.
The rape seed oil was obtained from Danisco A/S, Brabrand, Denmark.

Owing to food application of the produced MAG + DAGs a solvent free system was desirable. Moreover a solvent free system was optimal to keep a safe production environment and to avoid costly energy consuming solvent removal from the product mixture. However, attempts to obtain a solvent free glycerolysis system turned out unsuccessfully. The poor solubilization of the glycerol in the oil and high viscosity at low temperatures resulted in a heterogeneous system, low MAG yields and impractical operations. Hence, assistance of a solvent was needed to make the glycerolysis system homogenous, efficient and easy operated for industry. A major part of the experimental work was therefore centered on issues related to evaluation of the enzymatic glycerolysis system in an organic media. In Figure 6.2 is an overview of the MAG synthesis work with assistance of a solvent media given.

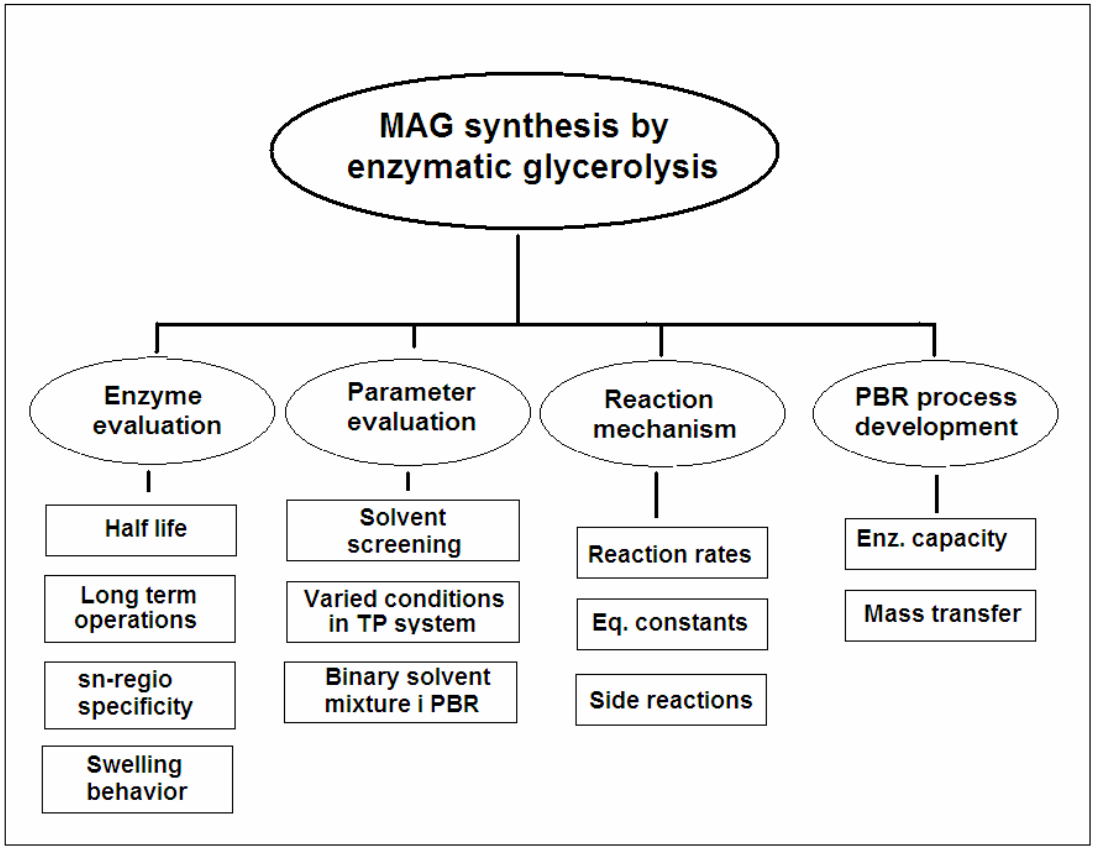


Figure 6.2: An overview of the experimental work performed related to MAG synthesis.

6.3.1 Enzyme evaluation

Novozym®435, supplied by Novozymes A/S, Bagsværd, Denmark, was employed as the biocatalyst in all the glycerolysis experiments. It was an immobilized form of *Candida Antarctica* B lipase (CALB) produced by submerged fermentation of a genetically modified *Aspergillus niger* microorganism. The carrier, physical bonded to CALB, was an acrylic-type macroporous support based on a hydrophobic matrix of poly methyl methacrylate (PMMA) (Lewatit VP OC 1600). The porous spherical beads structure were characterized by a bulk density of 430 kg/m^3, particle diameters of 300-900 μm, surface areas of 80-150 m^2/g and pore diameters of 140-170 Å (10^{-10} m) (Nakaoki *et al,* 2005; Novozymes A/S, 2001). The product sheet for Novozym®435 can be found in Appendix III.

Novozym®435 was selected as this has previously been demonstrated to have a unique qualification in the glycerolysis system (Yang *et al.,* 2005b & Kristensen *et al*, 2005). High catalytic effect was observed in a tert-butyl alcohol medium without

any water addition (Yang *et al.,* 2005a). This feature was of great importance to avoid undesirable hydrolysis of the acylglycerols to FFA. A comercially available enzyme was needed to obtain easy accessibility to a uniform and consistent enzyme supply to large scale plants. Although Novozym®435 is quite expensive compared to many other comercially available enzymes, the hydrophilic lipase in combination with a hydrophobic carrier material made it suited to bind glycerol as well as oil. Furthermore, a lipase immobilized onto a solid support material was desirable to have a robust and stabile catalyst suitable for long term continuous processing.

The enzyme swelling was investigated during wetting with the solvent reactant mixture (Paper IV). The sn-specificity of the lipase was investigated in batch as well as PBR operations by evaluating the isomeric forms of the produced MAGs and DAGs. Large amounts of reactants dissolved in TP and TB:TP mixture were reacted in enzyme PBR to evaluate the stability and half life of the Novozym®435 in these solvent systems. The reaction ran continuously (42 to 48 days) by renewing the reactant mixture reservoir when needed (Fig 6.3). Further details about the set up can be found in Paper IV.

Figure 6.3: Illustration of the set up for long term glycerolysis reaction in a PBR.

6.3.2 Parameter evaluation and optimization

Several pure and mixed solvents of varying polarities (log P values) were screened to evaluate the reaction performance of the enzymatic glycerolysis in different solvent systems. Since purified MAG was the product of major interest from the glycerolysis reaction, the system was optimized with respect to maximal MAG

contents. Solvents with different log p values (as defined in paragraph 5.1), were chosen as a useful way of comparing solvent properties to reaction performance for the lipid and lipase system. Table 6.4 gives an overview of the solvents included in the solvent evaluation, either used in their pure form or in binary mixtures. All the tested solvents were highly purified analytical grade chemicals with a purity of minimum 96%.

Table 6.4: Overview of solvents included in the screening sort by polarity (log P value) and corresponding melting /boiling point. Ref. Lide, 2007a; Lide, 2007b; KBA, 2007

Pure solvent	**Log P value**	**m. p. (°C)**	**b. p (°C)**
Acetonitrile	-0.34	-43.8	81.7
Ethanol	-0.30	-114.1	78.3
Acetone	-0.24	-94.7	56.1
Isopropanol	0.05	-88.5	82.4
2-butanone	0.29	-86.6	79.6
Tert-butanol	0.35	25.7	82.4
3-pentanone	0.82	-39.0	101.0
Tert-pentanol	0.89	-9.1	102.4
Chloroform	1.97	-63.4	61.2
Toluene	2.73	-95.0	110.6
n-hexane	4.00	-95.4	68.7
n-heptane	4.50	-90.6	98.4
Iso-octane	5.15	-107.0	99.2

The solvent effect on reaction performance, solubility, costs and melting and boiling point was tested in Batch (Paper I) as well as PBR operations (Paper III). The use of UNIFAC, a thermodynamic model (Hansen *et al.,* 1991), was included to predict m.p. and b.p. for certain binary solvent mixtures. These predicted values were compared to experimentally determined values by the use of Differential Scanning Calorimetry (DSC) (Paper III). UNIFAC was also used to predict the solubility of glycerol and oil in some of the solvents. Investigations of the phase split behaviour of glycerol and oil in the most potential binary solvent mixture was conducted to obtain a better understanding of a potential co-solute effect in the enzymatic glycerolysis system (Paper III). A three-level four-factor fractional experimental design with five star points (29 experiments) was conducted for enzymatic glycerolysis in TP to evaluate the effect of multiple independent factors and their interactions (Paper II). The factors and ranges selected were based on the results from studies by Yang & Xu, 2003 and preliminary screening tests and are summarized in Table 6.5.

Table 6.5: Overview of included factors and ranges for glycerolysis optimization in TP-system by the use of RSM.

Factor	Range
Enzyme dosage (wt% of oil)	5-10-15-20-25
Solvent amount (vol%/wt of oil)	200-300-400-500-600
Glycerol to oil ratio (mol/mol)	3-4-5-6-7
Reaction time (min)	30-60-90-120-150

6.2.3 Reaction kinetics

In the present project it was found useful to explore the overall enzymatic glycerolysis mechanism from a simplified and practical-oriented approach by focusing on the reactant and product concentrations of MAG, DAG, TAG and gly. Several reactions were considered included in these investigations. However, many equations were refrained from being included to maintain a simple and defined system. From the glycerol to oil optimization it was shown that optimal MAG yields were ensured by excess of non-reacted glycerol. Thus, the reaction mechanism between glycerol and each of the individual lipid components MAG, DAG and TAG were chosen to experimentally illuminate the forward reaction kinetics of equation 6.2 to eq. 6.4 with K_1-K_3 representing the equilibrium constants.

$$\text{TAG + gly} \xrightarrow[K1]{} \text{DAG + MAG} \qquad (6.2)$$

$$\text{DAG + gly} \xrightarrow[K2]{} \text{2 MAG} \qquad (6.3)$$

$$\text{2 MAG + gly} \xrightarrow[K3]{} \text{DAG + 2 gly} \qquad (6.4)$$

Well aware that Eq. 6.4 is of inconsiderable size compared to Eq. 6.2 and 6.3 it was included as a ‘substitute’ for the reverse Eq. 6.3 reaction in a ‘realistic glycerolysis invironment’ with excessive amounts of glycerol being present. Pure MAG, DAG and TAG raw material produced from unhardened rape seed oil was used for these experiments. For each of these individual forward reactions (Eq. 6.2-6.4), initial reaction rates and equilibrium constants were calculated as:

$$-r_{TAG} = \frac{d[TAG]}{dt} = [TAG]_t - [TAG]_{t=0\ min} = \frac{mol}{kg \cdot h} \qquad (6.5)$$

$$-r_{Gly} = \frac{d[Gly]}{dt} = [Gly]_t - [Gly]_{t=0\ min}$$

$$r_{DAG} = \frac{d[DAG]}{dt} = [DAG]_t - [DAG]_{t=0\ min}$$

$$r_{MAG} = \frac{d[MAG]}{dt} = [MAG]_t - [MAG]_{t=0\ min}$$

$$K_1\ (eq) = \frac{[MAG]_{t=eq} \cdot [DAG]_{t=eq}}{[TAG]_{t=eq} \cdot [Gly]_{t=eq}} \qquad (6.6)$$

$$K_2\ (eq) = \frac{[MAG]^2_{t=eq}}{[DAG]_{t=eq} \cdot [Gly]_{t=eq}}$$

$$K_3\ (eq) = \frac{[DAG]_{t=eq} \cdot [Gly]_{t=eq}}{[MAG]^2_{t=eq}}$$

A more detailed description of the experimental set up can be found in Appendix II.

6.2.4 Process development in PBR

A PBR set up was developed for continuous enzymatic glycerolysis. The principle of the set up is illustrated in Fig. 6.4 and includes a reactant mixture assisted by solvent pumped through an enzyme packed column and collected as product mixture.

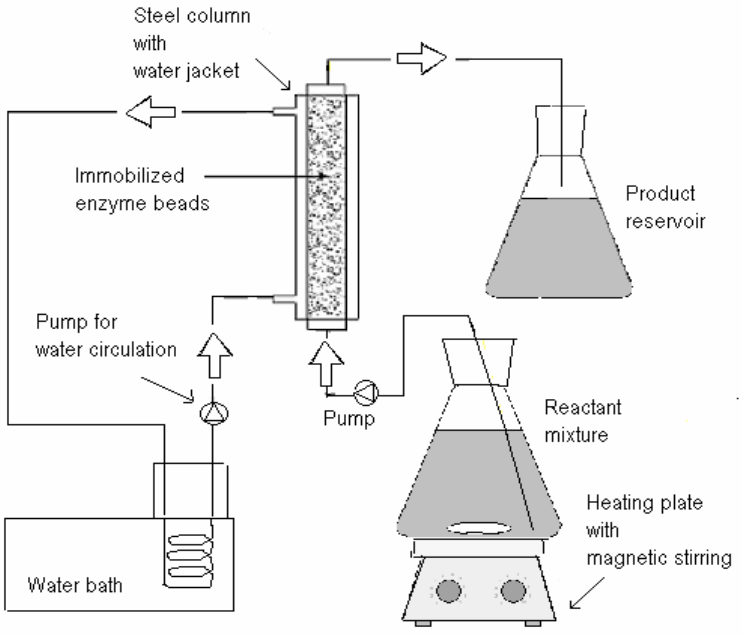


Figure 6.4: Illustration of continuous glycerolysis in PBR.

Several aspects such as different particle sizes of the enzymes, enzyme filling amounts, reaction times, flow rates and column length-to diameter ratios were tested. These parameters were selected based on an interest to experimentally investigate plausible mass transfer limitations and obtain an efficient and easy operated PBR set up. An overview of the parameters and the range selected are summarized in Table 6.6. More detailed desciptions can be found in Paper IV.

Table 6.6: Overview of investigated parametersand range for development of a continuous PBR set up.

Paramter	Range
Enzyme filling amount (kg/m^3 column)	114-227-340
Reaction time (min)	10-15-20-25-30-35-40
Average particle size of enzyme (µm)	388-548
Flow rates (ml/min)	0.95-1.8-3.5-4.6-11.6
Length-to diameter ratio of column	9.5 -26.7

6.3 MAG purification

The assistance of a solvent media in the MAG synthesis made subsequent solvent removal from the product mixture necessary. Separation of MAGs from the remaining product mixture was also desirable to obatian MAG in a pure form with minimal volume of unreacted glycerol, produced side-products as well as DAG and plausible TAG components. A stripping process and SPD was included in the MAG purification work to take advantages of the current industrial competences in these technologies. Membrane ultrafiltration was included as a potential low temperature alternative.

6.3.1 Rotary evaporation

The solvent was removed from different glycerolysis product mixture by evaporation under vacuum at different selected temperatures. A weighted sample amount was heated in a water bath during rotation. The evaporated components from here was then condensed by cooling water and collected in a vessel. The solvent/by product collected was hereafter calculated as wt% of the initial sample amount in the sample (Paper III).

6.3.2 Steam stripping for solvent and glycerol removal

Approximately 50 L product mixture obtained from long term continuous enzymatic glycerolysis reaction in a TP system (Paper IV) was stripped for solvent in a pilot plant facility at Danisco A/S. This was done to evaluate the possibility to combine solvent and glycerol removal in a process similar to the one currently employed at chemical glycerolysis. During the solvent stripping, the glycerolysis product mixture was heated to 160°C and feeded to the top of a stripping column under vacuum (25 mbar). The stripping column had an internal diameter of ø200 mm and was packed with 1000 mm structural packing material (Mellapack Optiflow). Steam was led into the bottom of the column and stripped of TP and other volatile components from the feed. The vapor was led to an external condenser and condensed at 20°C collected as distillate. The remaining mixture was hereafter analyzed for prospective solvent by evaporation experiments in laboratory.

6.3.2 Solvent and glycerol removal through membrane filtration

A preliminary study of the applicability of membrane ultrafiltration to remove solvent and glycerol was tested by separation of the glycerolysis product mixture through four different available membranes (Table 6.7)

Table 6.7: Characteristics of the membrane used for ultrafiltration.

Membrane	Characteristics		
	Selective layer	Support layer	Cut-off (M_w)
ETNA01PP	PVDF[a]		1,000
ETNA10A	PVDF[a]	PP	10,000
GR81PP	PSf	PP	10,000
Cellulose acetate			10,000

PSf= polysulphone, PVDF=polyvinylidenefluoride, PP=polypropylene, [a]Hydrophillic coated

The filtration was conducted in a stirred dead-end ultrafiltration cell with magnetic stirrer, provided from Millipore, Glostrup, Denmark, in an experimental set-up illustrated in Fig. 6.5.

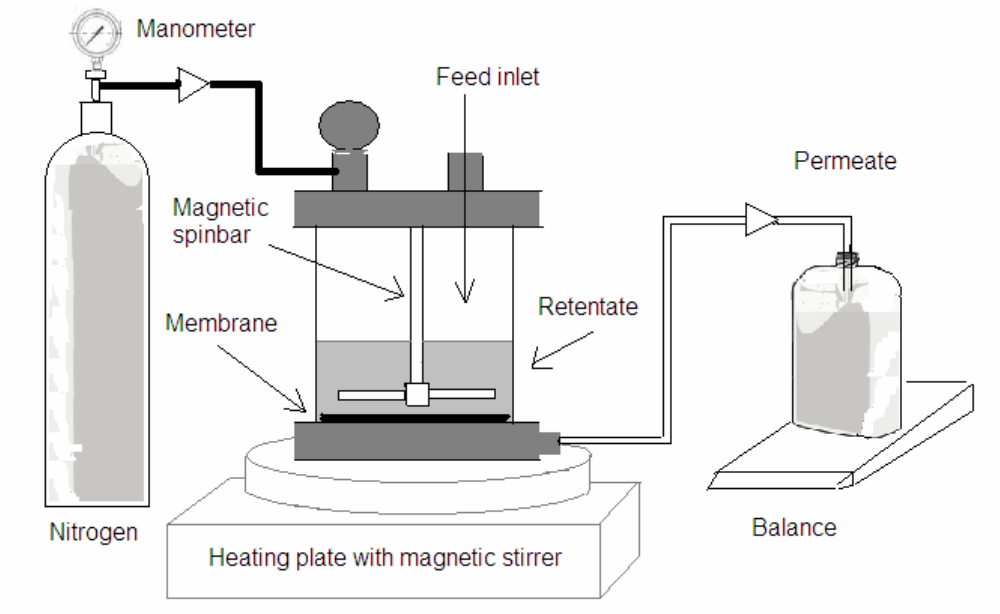


Figure 6.5: Illustration of MAG purification through membrane filtration. Adapted from Vikbjerg et al., 2006.

6.4 Up-scale experiments in pilot plant

To make first up-scaled trials, the developed small scale process in PBR was transferred to pilot plant equipment having reactor volumes 50 to 400 times bigger. Rape seed oil, supplied by ADM Hamburg AG, Hamburg, Germany was used as TAG raw material in these trials. Two long thin steel columns with large length-to-diameter (l/d) ratios were available, termed 'PBR I' and 'PBR II'. To supplement these very narrow columns containing a high enzyme bed a filter based column comprising a much wider and shallower enzyme bed, termed 'FILTER' was included. Fig 6.6 shows a picture of one of the long thin steel columns used (PBR I) and the Filter based set-up (FILTER) is illustrated by its principle sketch in Fig 6.7.

Figure 6.6: Picture from the PBR set in pilot plant facilities.

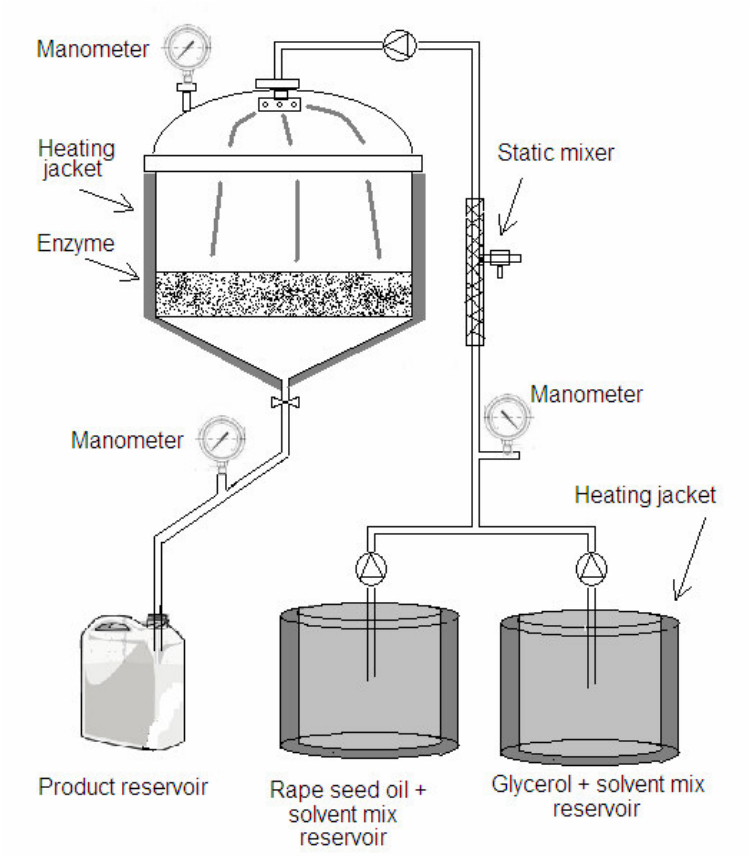


Figure 6.7: Principle sketch of the filter based set up conducted in pilot plant facilities.

The detailed equipment dimensions and reaction conditions for each set up are summarized in Table 6.8.

Table 6.8: Equipment dimensions and reaction conditions for enzymatic glycerolysis in different set-up.

Experiment ID	Equipment dimension			Reaction parameters					
	Dia (*d*) cm	Height (l) cm	Cross section column cm^2	Reac time (τ) min	Flow Glyce. kg/h	Flow Oil kg/h	Fluid Velocity[a] (υ) m/s	Wet enzyme[b] cm^3	Estim. height enz.bed[c] cm
PBR-I	4.7	100	17.3	10	1.42	3.37	$8.63 \cdot 10^{-4}$	1719	99
				20	0.71	1.69	$4.31 \cdot 10^{-4}$		
				30	0.47	1.13	$2.88 \cdot 10^{-4}$		
PBR-II	7.2	100	40.7	10	3.33	7.92	$8.63 \cdot 10^{-4}$	4032	99
				20	1.67	3.96	$4.31 \cdot 10^{-4}$		
				30	1.11	2.64	$2.88 \cdot 10^{-4}$		
FILTER	30		706.9	10	3.33	7.92	$4.97 \cdot 10^{-5}$	4032	6
				20	1.67	3.96	$2.48 \cdot 10^{-5}$		
				30	1.11	2.64	$1.66 \cdot 10^{-5}$		

[a]Calculated as flow $_{total}$/cross section of the column, with a density set to 1.0 kg/l for the glycerol-solvent mix and 0.85 kg/l for the oil-solvent mix.
[b]Calculated as the weight of dry enzyme multiply a volume/wetting expansion factor of 4.5.
[c] Calculated as wet enzyme/cross section of the column

7. Discussion of main findings of experimental work

A condensed description and discussion of the main findings obtained from the experiments conducted are given for each series of studies.

7.1 Evaluation of the TLC-FID methodology

A careful evaluation of the TLC-FID methodology is detailed described in Appendix I why the overall findings just are briefly summarized in the following. From screening of solvent suitable to single step separation of glycerolysis components (MAG, DAG, TAG and FFA) a n-heptane 35 mL: diethylether 35 mL: acetic acid 1 mL mixture turned out to be the most qualified combination. It showed a clear and sharp peak separation as illustrated in Fig. 7.1.

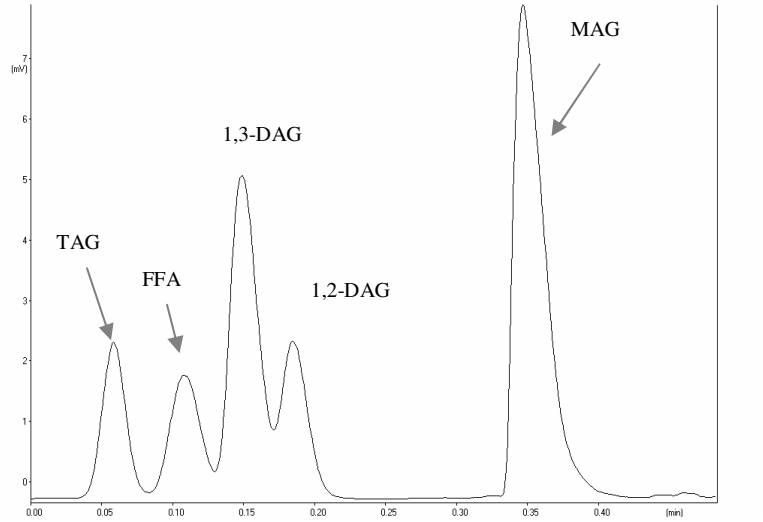


Figure 7.1: TLC-FID chromatogram obtained from a mixture of: 10,1 mg internal std 2 and 1,1 mg/mL free fatty acids separated by n-heptane 35 mL: diethylether 35 mL: acetic acid 1 mL.

Although this solvent mixture contained diethyl-ether, associated with flammable and harmful risks, it was proved impossible to substitute with non-toxic alternatives and still maintain a clear and sharp peak separation. The most potential alternative was substitution of the ether with 2-propanol and increasing the volume of n-heptane. However, this 'alternative' solvent combination resulted in overlapping peaks at broad concentration ranges which pointed to undesirable analytical limitations.

The use of standards demonstrated some difficulties with consistent measurements when pure compounds were mixed and the concentration was varied. Good linearity was obtained from the pure standards tested individually (mono-di and triolein). However, the obtained regression lines from a mixture of pure MAG, DAG and TAG standard varied from the single components tested. In the mixture of pure standards, a high uncertainty degree up to 15% between the measured and calculated peak area were observed. Thus, the standard curves were not believed suited for sample quantification. Instead the use of of propylgallate as IS was considered. The calculated response factors from here varied not only depending on the compound measured but also depending on the concentration of the individual components. Hence, semi-quantification from an IS was also believed inappropriate due to the inconsistency. In contrast, a non-quantitative approach based on a normalization of the areas per peak was found reliable at estimating the acylglycerol distribution. By this method, the relative lipid distribution of MAG, DAG and TAG could be estimated within an accuracy $\geq 90\%$. This non-quantitative method was believed very suitable for rapid and simple detection of the acylglycerol distribution from the actual glycerolysis samples.

7.1.1 TLC-FID versus the GC-FID method

In the actual process development, the reaction mixture was considered as a whole, in which the glycerol amount also needed to be paid attention (supplementary to the MAG, DAG and TAG content). Although attempts were made to include a separated glycerol peak in the TLC-FID chromatogram it failed when a clear separation of the acylglycerols had to be maintained. In contrast, the GC-FID method adopted from Danisco A/S was capable of quantifying all major components in the samples and also some minor compounds. Furthermore, the GC-FID method has the ability to identify different sn-regio specific isomer (1-(3)-MAG versus 2-MAG) and the chain length of the fatty acids, all in the same run (Fig. 7.2).

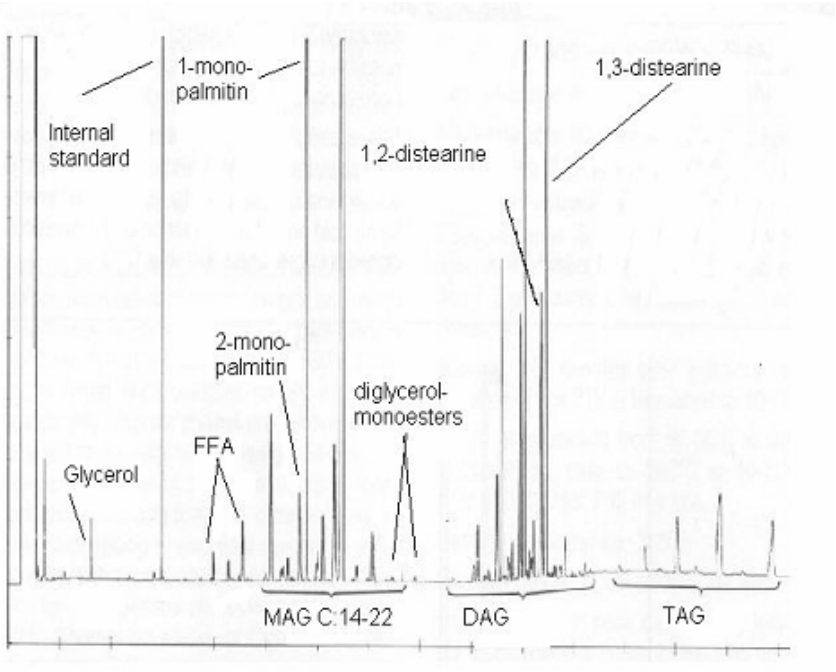


Figure 7.2: An example of a chromatogram obtained from GC-FID analysis of a glycerolysis sample.

The use of GC-FID was therefore found optimal when detailed information was needed. Normalization of comparable compounds from identical samples analyzed by TLC-FID (area%) and GC-FID (wt%) agreed very well (Fig 7. 3).

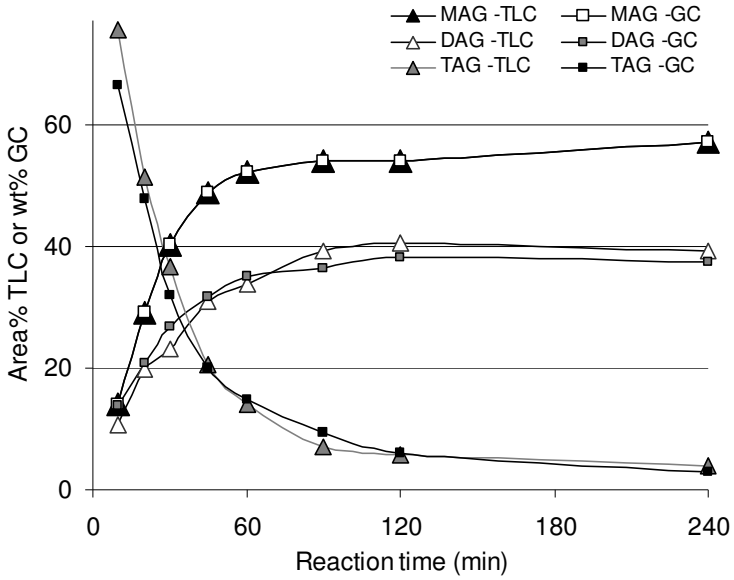


Figure 7.3: Identical samples analysed by TLC-FID (area%) and GC-FID (wt%)

This verifies the applicability of the TLC-FID method to precisely estimate the acylglycerols distribution based on the peak areas. Hence, all things considered, TLC-

FID was believed suitable for rapid detection of the lipid distribution while GC-FID was recommended when more details was required.

7.2 MAG synthesis by enzymatic glycerolysis

7.2.1 Screening of organic media

Although a solvent free system was most beneficial from a safe point of view it was proven impossible to find competitive alternatives to the solvent system. Addition of small amount of emulsifying purified MAG (5wt% DIMODAN) or lecithin (2 wt%) and strong agitation were tested. However these 'solvent free atempts' failed to improve the reactant homogeneity and MAG formation dramatically. Hence, solvent addition was believed necessary to overcome the immiscibility of the glycerol and oil, to reduce the reactant viscousity and to improve the transfer to the active lipase site. Usage of organic solvents is in contrast to the general principle of a 'white industrial biotechnolgy' but re-use of the solvent, which minimize the waste amount, made it reasonable to use anyway. The aspects related to solvent issues are detailed evaluated in Paper I-III. Here follow a summary of the major findings and considerations.

From a careful screening of the obtained MAG content after enzymatic glycerolysis in varied pure solvents with different polarity, especially TB and TP turned out very qualified (Paper I). These two solvents had tertiary alcohols structure (Fig 7.4) and 'medium polarity' with log P values of 0.35 and 0.89 for TB and TP, respectively (KBA, 2007).

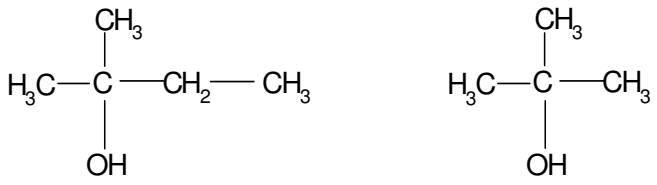


Figure 7.4: Tert-pentanol (2-methyl-2-butanol) and tert-butanol (2-methyl-2-propanol).

The 'medium' polarity with a combination of a polar –OH group and a non-polar carbon unit (Fig. 7.4) seemed to provide the required ability to 'hold' oil as well as glycerol. By that a dramatically improved reaction efficiency was obtained. This is

most likely a combination of better reactant mixing with greater oil/gly interface, reduced reactant viscosity and an activating effect on the lipase by which the reactants easily accesses the active site. However, other solvent tested with similar log p values like 3 pentanone did not lead to similar high reaction efficiency. Hence not only the log p value but also the structure of the solvent apparently influenced the reaction efficiency. The tertiary alcohol structure seemed sterically hindered towards interaction with the *C. Antarctica* lipase, confirmed by Yang *et al*. (2005a). Hence, TB and TP function as wellsuited inert carrier materials that not are involved in the reaction themselves. In contrat, the other alcohols tested such as ethanol and isopropanol showed inefficient reaction (Paper I). This is ascribed competitive alcohol interactions with the lipase resulting in restricted access of the glycerol to the active lipase site.

Previous investigations (Yang & Xu, 2003) have demonstrated difficulties in the practical handling of the pure TB media. Here, solvent removal and product purification by traditional distillation/condensation techniques was rendered difficult. Owing to a melting and boiling point for TB that are quite close (57°C) and a high melting point (25°C) it involved crystallization during condensation of the evaporated solvent. Solvent crystallization in the equipment makes continuous operations difficult and limits the possibility for readily reuse of the solvent. Hence, from an industrial point of view, the use of TB in its pure form has some drawbacks although it benefits from low cost and high obtained MAG content (Table 7.1).

Table 7.1 Overview of potential solvent for enzymatic glycerolysis with respect to practical operations in industry and low costs

Solvent	Solvent ratio vol/vol	Measured by DSC[a]	Predicted by UNIFAC method[b]		Literature values[c]		Price index[d]	MAG content[e] wt%
		m.p. (°C)	b.p (°C)	m.p. (°C)	b.p (°C)	m.p (°C)		
TB	100	21.9±2.2	82	24.9	82.2	25.5	107	54.8
TP	100	-12.0± 0.6	101	-9.1	103	-9	235	55.9
TB:TP	90-10	15.1± 1.4	83	17			120	46.6
TB:TP	80-20	8.8±0.9	85	7			132	55.4
TB:nH	80-20	10.9±0.6	72	10	73		144	45.4
TB:cH	80-20	7.9±0.5	77	7	75		100	45.6

TB=*tert*-butanol, TP=*tert*-pentanol, nH=*n*-hexane, cH=cyclo-hexane. [a]Differential Scanning Calorimetry; [b]Ref. Hansen *et al.,*1991; [c]Ref. Lide, 2007b; Govindaswamy *et al.,* 1977; Reddy & Rao, 1965; [d]Calculated as relative price index between solvent amounts of 5 tons with purity of 99% for TB, nH and c-hexane and purity of 98% for TP. Prices are obtained from Danisco A/S, Brabrand, Denmark (2005); [e]Obtained from continuous glycerolysis in PBR.

Even though, the cost of TP is almost 50% higher compared to the cost of TB, TP was considered as a reasonable alternative to TB. TP has a broader range between m.p. and b.p. (Table 7.1) which can be utilized to improved practical operations. The high reaction performance was maintained in the TP system in contrast to other screened pure solvents. Due to these useful operational properties, the enzymatic glycerolysis in TP was optimized by RSM in batch operations. From that experiment the enzyme dosage and reaction time showed a significant effect of the MAG formation while the effect of substrate amount and glycerol to oil ratio was insignificant in the range tested (Paper II).

To overcome the drawback of TPs high costs and TBs inconvenient melting-boiling profile, partial TB substitution seemed to be an even better alternative (Table 7.1). A melting point below approximately 15°C and a temperature range between melting and boiling point of at least 65°C was deemed sufficient to avoid practical problems with crystallisation. M.p. and b.p. for selected binary solvent mixtures were predicted from UNIFAC calculations and compared to experimentally measured m.p. by DSC and literature values, whenever it was possible (Table 7.1). Good agreement was found between predicted, measured and values reported in the literature. Replacement of 10-20 vol% of the TB with either TP, nH or cyclo-hexane (cH) seemed appropriate to obtain sufficient range between melting and boiling point, maintained high reaction efficiency and low costs (Table 7.1)**.** The changed m.p and b.p. profile for TB:TP 90:10 vol% compared to pure TB was only minor. Hence, this mixture still has a considerable risk of crystallization during downstream processing. Thus, with respect to the practical operation at industrial facilities, only binary mixtures with no more than 80 vol% TB seemed useful.

Highly flammable and harmful risks are related to all the four solvents included in the preferable binary mixtures (TB, TP, cH, nH) (Table 7.1). According to EU standards the maximal exposure of the four solvents are limited to: TB: 50 ppm; TP: 100 ppm; cH: 50 ppm and nH: 25 ppm. Thus, special precautionary handling procedures should be taken when working with these solvents. Good suction is required to avoid inhaled vapour solvents and gloves should be worn to avoid direct skin contact and accumulation in tissues (KBA, 2007*;* Stoye, 2008; Hahn *et al*., 2008; Lappe & Hoffmann, 2008). nH has the advantage of being accepted as food–grade

extraction medium by the international Food and Nutrition Board, supported by the U.S. FDA (FNB, 2004). However, the very low boiling point of nH at 68.7°C has the disadvantage of potentially being flammable and release harmful fumes at industrial facilities maintained at typically temperatures of 20-25°C. cH has better acceptance in industry than nH, reflected by a doubling of the maximum exposure limit of cH compared to nH (KBA, 2007*;* Stoye, 2008; Hahn *et al*., 2008; Lappe & Hoffmann, 2008). This is due to cH higher boiling point at 80.7°C which leads to reduced risk for vapor relase at room temperature. This provides a 'safer' environment during practical handling.

Even though food-legalization aspects slightly favor the use of nH for partial substitution of TB, it is difficult to recommend one solvent over the others based on hazard considerations only. Comparison of industrial obtained prices shows that TB:cH 80:20 vol% was the most economical choice while TB:nH 80:20 vol% carried the highest costs. The price gap of 44% between the cheapest and most expensive solvent combination is of considerable commercial importance. Although, the TB:TP 80:20 vol% was amongst the 'expensive' binary mixtures it provided the most optimal reaction performance. Hence, it was found unreasonable to exclude any of the binary mixtures without further cost-benefit analysis with respect to factors such as space-time requirements compared to product yield.

Despite this, TB:TP 80:20 vol% demonstrated a great potential due to an attractive combination of high reaction performance, reasonable cost, easy handling in practical operations and 'high' exposure limit compared to the other binary solvent mixtures. Because of these desirable capacities, this TB:TP mixture was widely used in the further process development. Its impact on the reaction performance in PBR was evaluated at varied dosages, summarized in Figure 7.5.

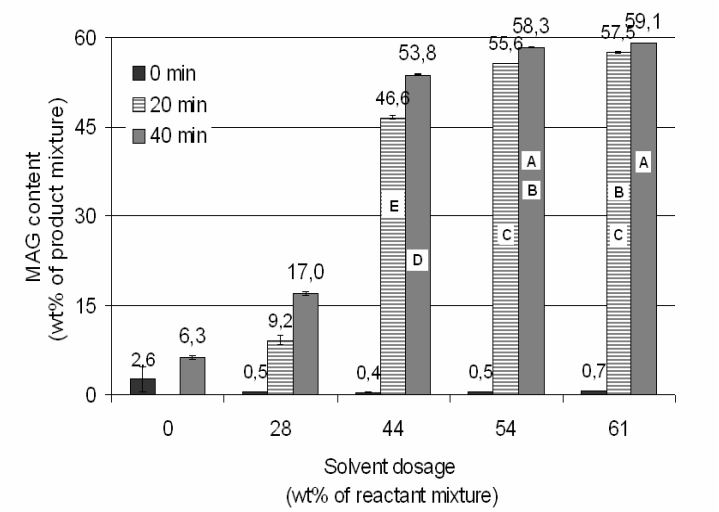


Fig 7.5: MAG content after continuous glycerolysis in a PBR at varied volumes of a binary mixture of TB:TP 80:20 vol%. The solvent free system was added 5 wt% DIMODAN (high purity MAG). Different letters (A-E) express a statistical significant difference.

A reaction time of 20 minutes and a solvent volume of 44% or 54% yielded similar and highest possible MAG contents per space time (Fig. 7.5). Prolonged reaction time and increased solvent dosage just lowered the MAG content per space time and increased the solvent cost, making these conditions less attractive from a comercially point of view (Paper III). The pattern with an insignificant effect of the 'high' solvent volume of more than 50 wt% agrees with findings for the pure TP system (Paper II). This implies that solvent used at these concentrations more or less are utilized to its full potential. A solvent dosage of 54 wt% is believed most attractive to obtain highest possible MAG content in the product mixture, while 44 wt% solvent has the benefit of lower solvent consumption**.** To obtain high purity MAG products subsequently purification processing is required which is believed similar for either 44 or 54 wt% solvent being present. Anyhow, a required solvent concentration of 44-54 wt% is regarded as a high dosage that also represents a major part of the raw material costs as illustrated in Fig 7.6.

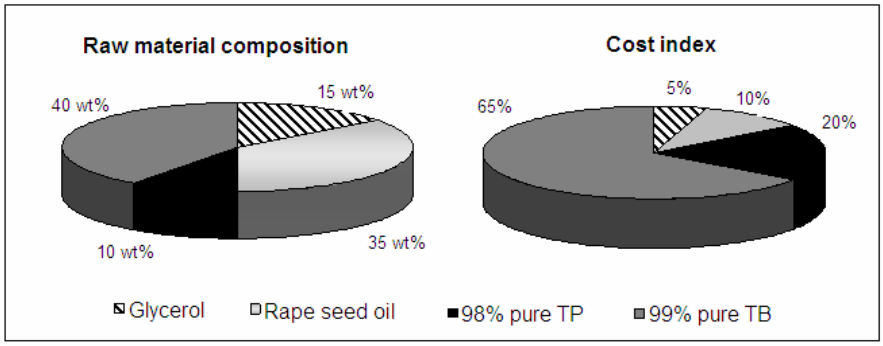


Fig 7.6: Raw material composition and cost for a suggested reactant mixture in organic media used for enzymatic glycerolysis. Prices are obtained from Danisco A/S.

The solvent represents as much as 85% of the total raw material cost assumed that 50 wt% TB:TP 80:20 vol% is used in the glycerolysis system, a glycerol to oil molar ratio of 4 is selected and the enzyme costs is excluded (Fig. 7.6). With such massive solvent costs it definitely emphasizes the importance of recycle considerations to make the solvent glycerolysis system industrial feasible/sustainable.

Pure triolein (TAG C18:n-1) and pure glycerol have been predicted to be completely soluble in TB at temperatures above 78°C and in TP above 28°C (Paper I). From this prediction, it was assumed that a binary mixture of TB:TP resulted in a homogeneous solubilized reaction mixture somewhere in the temperature ranges from 40°C to 100°C. However, in the TB:TP system, a two-phase system, with glycerol- and oil-rich phases, was surprisingly observed regardless of the temperature tested in the interval from 40 to 100°C. Thus, contrary to expectations, it was impossible to solubilize glycerol and oil in the TB:TP binary solvent mixture. Glycerol and sunflower oil was mixed separately with TB:TP 80:20 vol% to test whether this phase split occurred due to the immiscibility of either glycerol or oil. Results showed that glycerol as well as sunflower oil, in pure form, was completely miscible in the binary solvent mixture even at relatively low temperatures of 20°C. This confirms the predicted ability of the binary solvent mixture to dissolve polar as well as non-polar compounds. However, the observed phase split suggests a co-solute effect or indicates that the solvent mixture prefers one compound from the other when brought together. Hence, each phase was subject to a more detailed evaluation. Compressed results from the obtained phase split analysis by mixing, sunflower oil, glycerol and a binary mixture of TB:TP 80:20 vol% (Paper III) are listed in Table 7.2.

Table 7.2 Phase split behavior from mixing sunflower oil, glycerol and TB:TP 80:20 vol% at 75°C.

Compound	Weight wt%	Distribution measured		
		Upper phase wt%	Lower phase wt%	Total wt%
Oil	36.0	40.5	-	40.5
Glycerol	13.9	1.2	12.3	13.5
TB:TP mixture	50.1	44.6	1.4	46.0
Total	100	86.3	13.7	100

In the upper phase, the less dense oil accounted for 96-97 wt% while the denser glycerol only accounted for less than 4 wt%. In the lower phase, the opposite tendency was obtained with 90-100 wt% of the dense glycerol and less than 10 wt% of the oil situated (Table 7.2). The solvent distribution with more than 92% of the

solvent situated in the upper oily phase and less than 3.5% in the lower glycerol phase clearly indicates that the solvent mixture prefer oil compared to glycerol. This obvious phase split behavior does not suggest particularly improved reactant miscibility in the solvent system compared to the solvent free system. Instead, this suggests, that the enhanced reaction efficiency in the solvent system relates to other aspects such as lipase activation, reduced viscosity and changed system polarity and hereby better system stability. Although the two reactants apparently not were completely solubilized the presence of an organic medium provided a better 'emulsion' of glycerol and oil with greater surface area accessible for the lipasse. Changes in the polarity prevent adherent glycerol to the enzyme and hereby avoid a restricted contact between oil and enzyme. Reduced viscosity of the reaction mixture makes the flow and hereby the transfer to the active enzyme site easier with plausible mass transfer limitations being reduced. Finally, it should be noticed that the phase split behaviour was observed in the reaction mixture only. During reaction in PBR, the conversion of TAG and glycerol to more amphiphilic emulsifying MAG and DAG molecules presumably helps with enhanced homogeneity of the liquid passing through the column.

7.2.2 Suitable glycerol to oil ratios

The RSM optimization of the pure TP system showed surprisingly, an insignificantly main effect of glycerol to oil molar ratio from 3 to 7 (Paper II). Even so, the ratio between glycerol and oil is found to be of great importance for the acylglycerol distribution with more glycerol producing more MAG (Table 7.3).

Table 7.3: MAG to DAG ratio obtained from enzymatic glycerolysis performed at varied glycerol to oil molar ratios in three different organic media

Organic media	Gly:oil molar ratio before reaction	MAG:DAG Ratio (wt%)
TB	2.1	62:38
	2.6	68:32
	4.2	76:24
	5.1	79:21
TB:TP	0.1	7:93
	1.1	40:60
	2.1	61:39
	4.1	75:25
	6.1	80:20
TP	3	70:30
	5	80:20
	7	84:16

A high glycerol to oil molar ratio seemed very promising for high MAG formation after glycerolysis reaction (Table 7.3). However, these findings exclude the glycerol content that typically are represented in the equilibrium mixture due to the reversibility of the system (Krog, 1997). Hence, glycerol quantification is essential to be included when the optimal glycerol to oil ratio is selected. Results obtained from experiments including determination of the glycerol content is summarized in Fig 7.7a.

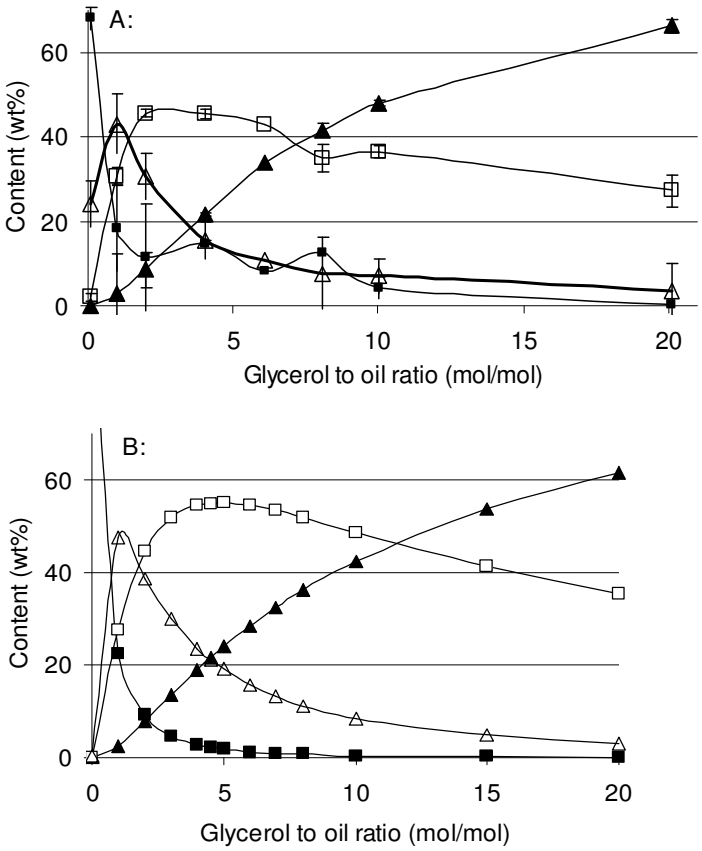


Figure 7.7: A: Experimentally determined compound distribution after glycerolysis in a tert-butanol: tert-pentanol 80:20 vol% system at a reaction time 180 min, compared to B: Theoretical estimated compound distribution after glycerolysis at equilibrium conditions, assumed that the ester formation follow a binomial random distribution with $p_{ester} = {}^{n}FA/{}^{n}OH$, *and glyceroll b(0\3;* p_{ester}*); MAG: b(1\3;* p_{ester}*), DAG: b(2\3;* p_{ester}*), and TAG: b(3\3;* p_{ester}*).* ■ *= TAG,* Δ*=DAG,* □*=MAG,* ▲*=Gly.*

At increased glycerol to oil ratio, the TAG conversion was enhanced like the amount of non reacted glycerol. The DAG formation reached a local optimum at a glycerol to oil molar ratio of two and the MAG formation reached a local optimum at five (Fig 7.7a). The findings were compared to theoretical estimated values based on a binomial random distribution of the FA (Fig 7.7b) and agreed well. Fig 7.7 shows that an increased glycerol to oil molar ratio is not solely beneficial for high MAG

formation and emphasize the importance of keeping strict in mind how the product mixture is evaluated when searching the literature. The actual process development was approached to obtain highest possible space time yield of MAG and a high TAG conversion close to 100%. These needs were fulfilled by a glycerol to oil molar ratio in the range from approximately four to six, yielding 54.5-55.1 wt% MAG and a TAG conversion of 97-99% (Fig 7.7). Hence, a ratio within this range was widely used in the further process development. Although, the MAG content obtained from this ratio range was considered high, major amounts of glycerol (19-29 wt%) and DAG (16-24 wt%) were present in the product mixture as well (Fig. 7.7).

A lower selected substrate ratio from two to four was considered very attractive for industrial usage too. By lowering the substrate ratio for instance to two, the MAG formation and TAG conversion was kept high, while the glycerol and DAG distribution changed in a way that was believed better utilized by the industry. A current used glycerol to oil ratio of two in industrial chemical glycerolysis, confirms the industrial benefits of this ratio (Krog, 1997). At this ratio approximately 8 wt% gly, 45 wt% MAG, 39 wt% DAG and 9 wt% TAG were estimated (Fig 7.7b) providing TAG and glycerol conversion degrees of 89 and 64 wt%, respectively. At this 'low' substrate ratio, the surplus of glycerol was decreased while the DAG amount was increased compared to the amounts obtained at higher ratios. Even though excess glycerol can be removed from the product mixture and re-used, it is regarded as an undesirable 'by-product' that takes up costly space. In contrast, the formed DAG is considered as a desirable 'by-product' that can be utilized in several ways. Pure DAG can for instance serve as main component in a relative new type of health improved dietary oil in combination with TAG (Flickinger & Matsou, 2003). MAG + DAG mixtures functions as emulsifiers and although such mixture contains considerably amounts of DAG they still fulfil the food grade emulsifier requirements of at least 70 wt% MAG + DAG, 30% MAG, and maximum 7 wt% glycerol (EFEMA, 2004). However, it is worth mentioning that MAG has better emulsifying properties than DAG why highly purified MAG products are of greatest commercial interest.

Another considered approach was to select a very high glycerol to oil ratio. By using a glycerol to oil molar ratio of for instance 20, a MAG:DAG:TAG ratio of

approximately 90:10:0 wt% was obtained from theoretical estimations as well as experimental determined values (Fig. 7.7). Excluded solvent and glycerol considerations, such high substrate ratio benefits from highly pure MAGs formed directly during the glycerolysis reaction. By this approach, a costly subsequent MAG separation from the other acylglycerols could be avoided. The current separation of MAG by SPD has for instance high energy consumption due to the high temperatures required. Furthermore, this extra processing step comprises a potential risk for product damage because of sensitive PUFAs being present. Hence, removal of a large glycerol surplus (62 wt%) might be more beneficial than including SPD processing or the like, even though the MAG space time yield is rather low (35 wt%). Anyhow, industrial cost-benefits analysis must be performed to find the optimized balance between product characteristics and the required processing.

7.2.3 Enzyme evaluation

The main findings from an evaluation of the sn-regio specific behavior of Novozym®435 in batch as well as PBR glycerolysis systems are summarized in Table 7.4. For further details please refer to Appendix II.

Table 7.4: Relative ratio between the different sn-positioned MAGs and DAGs based on fatty acid chain length (C16 & C18) after enzymatic glycerolysis in a TP medium.

Set up	Time (min)	MAG ratio wt% sn-1:sn-2		DAG ratio wt% sn-1,2:sn-1,3
		C-16	C-18	C-16 + C18
Batch	5	94:6	74:26	90:10
	240	86:14	89:11	34:66
PBR	10	91:9	83:17	70:30
	40	89:11	89:11	37:63
Long term PBR-1008 h	30	92:8		38:62
Chemical glycerolysis[a]		~ 90:10		~ 33:67

Ref: Laszlo et al, 2008; Flickinger & Matsou, 2003.

Overall, similar patterns were observed for the sn-distribution in the batch as well as the PBR reaction system (Table 7.4). A clear predominance of the sn-1 typed MAGs was formed during glycerolysis, accounting for 83 to 94 wt% of the total MAGs (Table 7.4). For the MAGs containing C-18 acyl residues (representing ≥ 90 wt% of the acyl residues in the oil used), a tendency to decreased sn-2 MAG contents were observed as the reaction time was prolonged (Table 7.4). This suggests an

inversion of the MAG molecules from the sn-2 to the sn-1 position as the reaction progresses. This is ascribed common acyl-migration where the acyl-group spontanuously migrates from the sn-2 position to the more stable sn-1 position (more detailed described in paragraph 2.2). However, totally opposite to that, MAGs comprising C-16 acyl residues (constituting approximately 5-7 wt% of the acyl groups in the oil) demonstrated increased sn-2 MAGs content as the reaction time was prolonged (Table 7.4). This hints on a cetain lipase behavior on specific chain lengthened acyl residues. However, since acyl group can be both cleaved of and attached to acylglycerols during the glycerolysis reaction the picture of an eventual regio-specific action of lipase during MAG formation is very unclear. For the DAG isomers a shift in the isomeric forms were observed as the reaction time was prolonged with a majority of 1,2-DAGs at short reaction times shifting to a majority of 1,3-DAG at longer reaction times (Table 7.4). This suggests acyl migration during prolonged reaction time but also a preference of the present lipase to initially act on the sn-1 (3) position during the TAG conversion. As expected, long term operations did not alter the different sn-ratios remarkably, indicating that no effect on the enzyme specificity was seen over time (Table 7.4). In all, an inconclusive picture of the sn-regio specific behavior of the lipase was observed due to plausible concurrent ester cleavage and formation and potentially interference from acyl-migration. Anyhow, the enzymatic glycerolysis provides MAG and DAG components distributed with 1-MAG:2-MAG ratios and 1,2-DAG: 1,3-DAG ratios close to common equilibrium ratios of ~ 90:10 and ~65:35, respectively (Kristensen *et al*, 2005; Laszlo *et al*, 2008; Flickinger & Matsou, 2003). Hence, independent of random or specific positioned lipase action it seemed impossible to affect the final regio specific distribution of 1-MAG, 2-MAG, 1,2-DAG and 1,3-DAG from enzyme catalyzed glycerolysis.

Typically, enzymes gradually loose activity over time due to deactivation/inhibition. To clarify the stability of Novozym®435 in the actual system long term operated glycerolysis were conducted in two organic media (Paper IV) (Fig. 7.8).

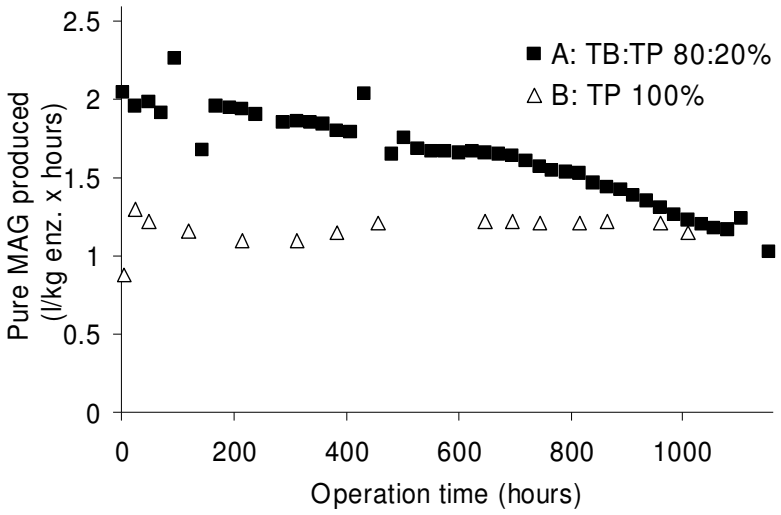


Figure 7.8 Time course for MAG produced pr. hour after long term continuous glycerolysis in A: TB:TP 80:20 v/v system (50 wt%) with reaction time of 20 and in B: pure TP system (51.4 wt%) with reaction time of 30 min.

In general, Novozym®435 showed consistent stability, capacity and long half-life in both solvent systems and maintained activity even after being used for 1,000 operation hours. Surprisingly, a considerably more stable TP system was observed in contrast to a clear linear time dependent decay in the TB:TP system. Although both systems were exposed to identical temperature, similar operation time, laminar running flows, same batch of enzyme, and substrate mixtures with same source of solvent (tertiary alcohols), the results imply different lipase inhibitory effects over time (Fig 7.8). TP only differs from TB by an additional carbon atom placed in the alcohol chain. This leads to a slight difference of polarity which might bring some difference of the enzyme activity. However, such effects were not noticed in early studies (Yang *et al.,* 2005a). Although different flow rates were used, both systems reached equilibrium MAG content (50-55 wt%) within the reaction time. The initial higher MAG production rate in the TB:TP system compared to the TP system, agreed with a 50% higher flow rate used. After exploring varios reasons, a higher total reactant mixture exposure in the TP:TB system (65.7 L) compared to the TP system (36.3 L) was the only major difference. This indicates a certain exposure limit of reactant mixture before a decrease in the enzyme activity occurs. With 90% residual activity set to the limit for an inhibitory effect this corresponds to exposure of approximately 3600 L reactant mixture/kg enzyme in the TB:TP system and 4500 L reactant mixture/kg enzyme in the TP system (Fig. 7.8).

In spite of the different stabilities in the two organic media: TP and TB:TP (Fig. 7.8), deactivation will happen over time. Conformational lipase changes, water deprivation of the enzyme, release of the carrier bond to the enzyme and disruption in the reactant mixture are all believed to be responsible for the reduced activity during prolonged operating time (Weete, 1998; Hayes, 2000). In the present set up, the inhibitory effct most likely comes from exposure to the reactant mixture which includes 'crude' sunflower oil and solvent both containg minor amounts of impurities. Assumed that the enzyme activity, in the TB:TP system, follows the ordinary first order deactivation kinetics, the life time of the enzyme was calculated to 2200 h (92 days) with a half-life set at 1200 h (based on estimation from Figure 7.8) and a lower limit for enzyme usage set to 25% residual activity (Paper IV). In comparison, alcoholysis of vegetable oil (for biodiesel production) demonstrated 84% retained initial activity of Novozym®435 after 9 cycles in batch reactor (of 7 hours) while Lipozyme®TL IM (comercially available *T. lanuginosus* lipase immobilized to an acrylic resin) demonstrated only 10% initial activity after one cycle (of 24 hours) (Hernández-Martín & Otero, 2008). This implies together with the long estimated life time suitability of Novozym®435 for long term continuous operations with relatively seldom catalysts replacement during processing.

A simplified enzyme packing of the column reactor is attractive for practical reproduceable operations. Hence, to minimize time consumption and keep the operating procedures simple pre-treatment and addition of a filling material was tried avoided at the reactor preparation. 'Dry' enzyme was poured directly into the column, as supplied from the manufacturer. Although this 'packing method' worked sufficiently, the subsequent column stabilization, by running reaction mixture through the column, led to a pronounced expansion of the volume. We observed a 92% volume increase as the enzyme went from dry to wet condition (Paper IV). According to literature, the used enzyme carrier material: PMMA is known to swells in some organic solvents (Kaneda & Vincent, 2004; Ruzzu & Matthis, 2002). Hence, in the tertiary alcohols media, the PMMA carrier material was believed responsible for the observed swelling. Our results showed that the expansion of the immobilized enzyme was independent of time (0.5 to 24 hours). This indicates that swelling saturation of the enzyme was ensured fast (paper IV). Thus, initial wetting of the dry enzyme bed

could easily be done directly in the column in reasonable time before the actual glycerolysis reaction was started.

The observed swelling emphasize the importance of avoiding an enzyme overload in the column to minimize problems with pressure drop and physical blocking in a PBR set up (Zhang, 2004; Xu *et al.,* 1998). On the other hand, it was desirable to add sufficient enzyme to obtain a 'full' PBR after wetting to obtain most controlled flow, avoid plausible back-mixing and utilize the reactor volume in an optimal way. Hence, based on the found swelling, the optimal packing of the column was believed to be at an enzyme amount of 227 kg dry enzyme/m^3 column. Experimental findings agreed with such enzyme amount being optimal (Fig 7.9).

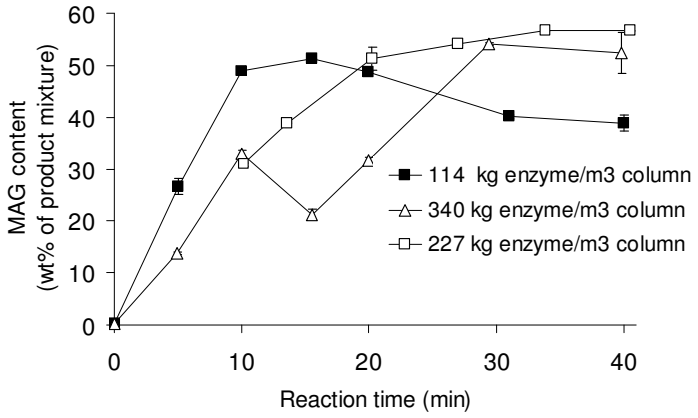


Figure 7.9 Measured MAG content after glycerolysis at different reaction times and varied enzyme loadings in a TB:TP mixture. Error bars represent STD from double determinations.

A low enzyme filling degree of 114 kg enzyme/m^3 reduced the MAG formation. This is ascribed reduced enzyme contact due to plausible channel formation in the enzyme bed (Fig 7.9). A load of 340 kg enzyme/m^3 also resulted in reduced activity with hindered contact to the active site of the enzyme ascribed the tightly paked enzyme bed (Fig. 7.9).

The Novozym®435 were fractionized into 'small' and 'big' particles and used for continuous glycerolysis to investigate plausible internal mass limitations (Paper IV).

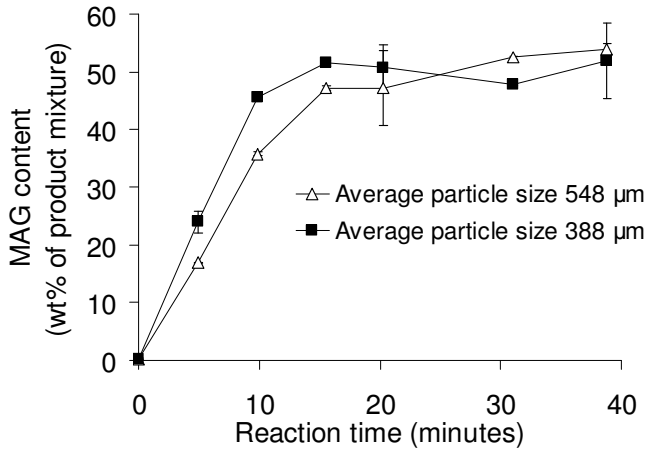


Figure 7.10: MAG content after glycerolysis in PBR by the use of two different sized enzyme particles.

Some differences in the MAG content were observed from glycerolysis performed with the small 388 µm particles compared to the bigger 548 µm particles (Figure 7.10). The initial reaction rate was for instance 30% higher for the 5 minutes reaction performed with the smaller particles as compared to the larger particles. Thus, the small particles seemed to enhance the diffusion of the reactant mixture through the pores to the active lipase site compared to the larger particles. This indicates the existence of internal mass transfer limitation for the large particles used. Such an observation is in accordance with the findings by Murty *et al*. (2004) for other lipase particles sizes (1 and 2 mm) used in the continuous PBR. However, we observed that the reaction rate between the different particle sized reactions converged as the reaction times were prolonged ending in similar reaction rates after 40 minutes (Fig 7.10). With an expected PBR reaction time of minimum 20 minutes to reach conditions close to equilibrium conditions (Paper IV), the differences in MAG content as well as reaction rates was not significantly marginal. Hence, differences between small and large particles were believed only minor in an overall consideration. Furthermore, the reduction of particle sizes of enzyme will in general increase the pressure drop, a very unfavorable properties for industrial applications. Thus, the lipase was believed adequate to use as it was with its comercially available size distribution from 300 to 900 µm.

7.2.4 Side reactions

From the detailed GC-FID analyses, minor amounts of free fatty acids (FFA) as well as fatty acid esters (FAE) were detected in the product mixture from enzymatic glycerolysis conducted in the TB and TP media, in Fig. 7.11 illustrated for the pure TP system.

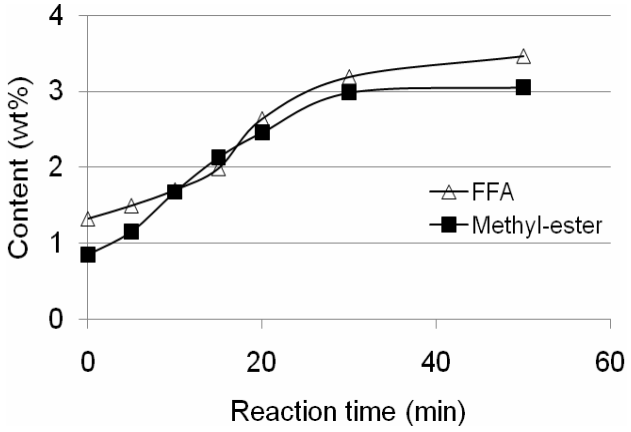


Figure 7.11: Time course for FFA and methylester formation during enzyme catalyzed glycerolysis in a TP system at glycerol to oil molar ratio of 5 in batch operations.

An increasing FFA and FAE formation of approximately two wt% were observed which happened synchronous to each other. The equilibrium amounts of FFA and FAE were reached faster than the MAG formation (after approximately 30 minutes compared to MAG that reached equilibrium after 70 minutes). A similar pattern was seen for enzymatic glycerolysis conducted in the binary TB:TP media (data not shown). Although a few wt% FFA are common from chemical glycerolysis (Krog, 1979) the presence of FFA and FAE were not expected to participate in the overall glycerolysis reaction (described in paragraph 4.1.1). FFA is a typically product from the hydrolysis reaction between oil and water (Chirsilp *et al.,* 2007) and FAE can potentially be produced by alcoholysis reaction between an acyl-group and alcohols (Xu, 2003). This indicates that plausible side reactions occur concurrently to the overall glycerolysis reaction. Therefore, major plausible side-reactions were considered for the present glycerolysis set up (Fig 7.12).

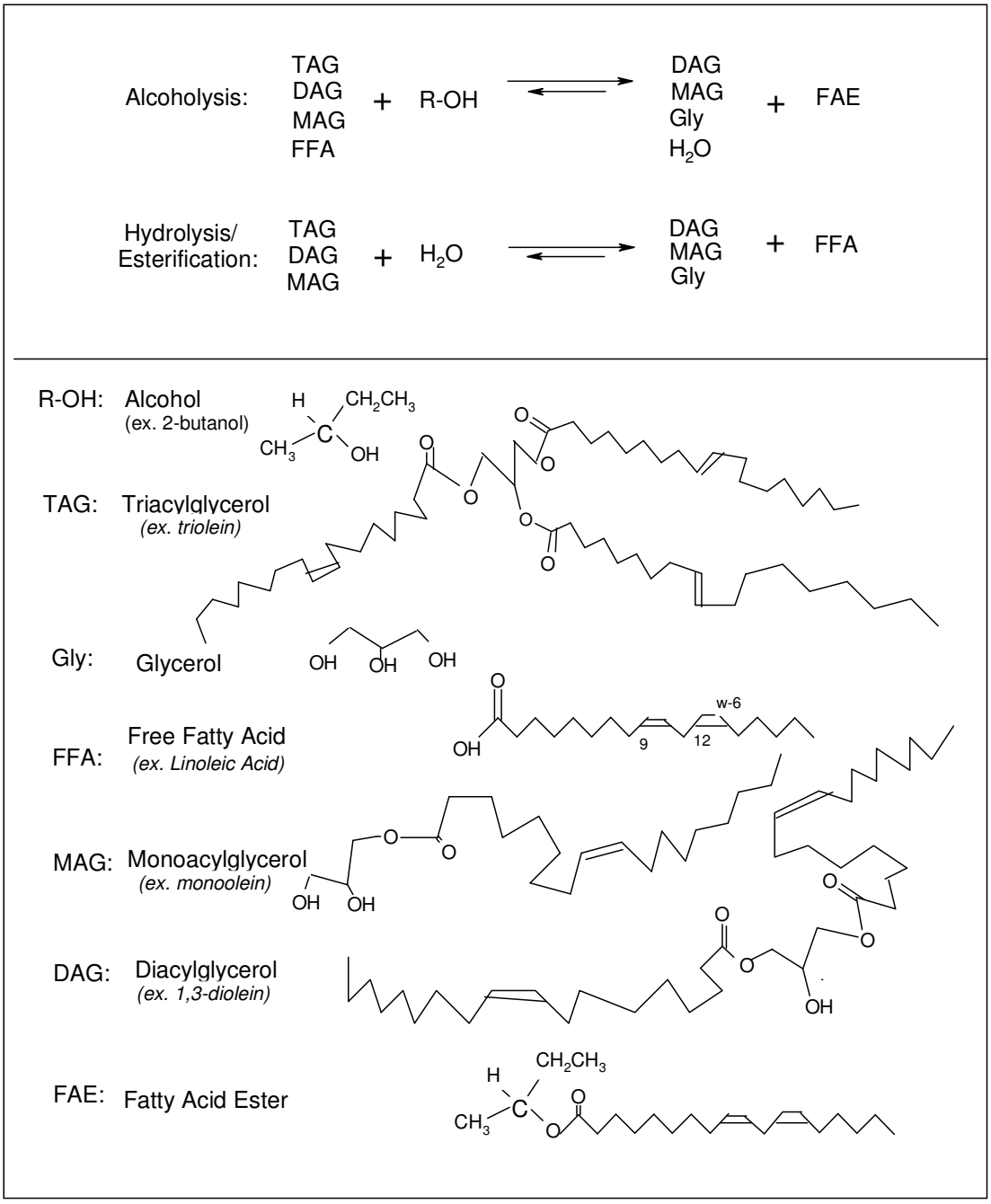


Figure 7.12: Illustration of plausible side reactions during enzymatic glycerolysis in a alcohol media and examples of the chemical composition of the compounds formed.

Besides the desirable alcoholysis between glycerol and either TAG or DAG, hydroxyl-groups (-OH) from other alcohols can react and give rise to an undesirable ester exchange to the alcohol (Fig. 7.12). Interference from the alcohols TB and TP is very conceivable since the organic media in general accounted for approximately 50 wt% of the system. The tertiary hydroxyl group (-OH) was believed steric hindered towards interactions with the *C. antarctica* lipase B confirmed by Yang *et al.* (2005a). However, some of the components less represented in the present system might

include OH-group available for FAE formation. Although highly purified TB and TP were used (96-99%), the remaining 1-4% was permitted to be other chemical components. Our examination of the TB and TP media using GC-MS analysis demonstrated presence of ethyl acetate ($CH_3COOCH_2CH_3$) and 2-butanol ($CH_3CHOHCH_2CH_3$) as impurities. Alcohols compete with glycerol as reactant for the alcoholysis reaction (Peng *et al.,* 2000) which implies that 2-butanol most likely is involved in the FAE formation. Results reported by Gulati *et al.* demonstrated high esterification degree between saturated C18-LCFA and primary-, secondary- and to some extent tertiary-alcohols (Gulati *et* al, 2003). Although unsaturated LCFA constituted the majority of the FA in the used rape seed oil (95 wt%) approximately 5wt% C-16 FA was present. Hence, it is very plausible that this minority of saturated FA is involved in the esterification reaction with the secondary 2-butanol. Thus, the observed FAE formation was believed formed by the combination of saturated FA and impurities in the TB or TP media.

Despite the strictly non-aqueous reaction system, the obtained FFA nevertheless implies water present in the system caused by plausible TAG, DAG and MAG hydrolysis reactions (described in paragraph 4.1.1, eq. 4.4-4.6). Even though the water content was measured to be less than 0.1 wt% for all materials used (Paper I) trace amounts of water from some of the components is plausible. Assumed that the 53-77 wt% solvent contain just 0.1% water, this water amount corresponds to 0.8-1.25 wt% formed FFA. Although these amounts only partly explain the detected FFA amounts (up to 5wt% in certain cases) it implies that only very small amounts of water are required for the unwanted hydrolysis reactions. The lipase enzyme requires water to maintain activity and its functionality in non-aqueous environments (Zaks, 1991). Hence, small amounts of water must be encapsulated in the carrier material together with the enzyme to keep it active. The volumetric Karl Fisher titration used to measure the water content, detects only ‘free’ water molecules. Thus it is conceiveable that a small amount of water was ‘hidden’ from detection. Hence, this ‘encapsulated‘ water most likely contributed to the FFA formation.

7.2.5 Reaction kinetics

A detailed discussion of the reaction kinetics can be found in Appendix II. The achieved equilibrium condition achieved from investigations of enzymatic glycerolyis conducted with glycerol and each of the three individual oil media: MAG, DAG and TAG are summarized in Table 7.5.

Table 7.5: Achieved equilibrium conditions after enzymatic glycerolysis for 240 min. in a tert-pentanol system conducted with TAG, DAG and MAG from rape seed oil.

Equilibrium conditions		Reaction medium		
		TAG + gly	DAG + gly	MAG + gly
Eq. content (wt%)	TAG ± STD	0.2 ± 0.1	-	-
	DAG ± STD	11.1 ± 0.2	9.8 ± 0.4	7.31 ± 0.2
	MAG ± STD	53.6 ± 0.1	54.0 ± 3.9	53.8 ± 1.5
	GLY ± STD	25.8 ± 0.1	28.5 ± 0.7	34.3 ± 0.6
Reached in (min)		70	30	10
MAG:DAG ratio		84:16	85:15	88:12
Eq. constants	K_1	65.28	65.36	-
	K_2	5.01	4.75	5.24
	K_3	0.20	0.21	0.19

All three investigated reactions converge towards similar MAG and DAG equilibrium content independent of the starting material (Table 7.5). The contents obtained at equilibrium implies that it is not feasible to push the reaction to only MAG formation without reverse reactions spontaneously occurring (Table 7.5). The initial amount of MAG or DAG present in the system seemed to have only a minor effect on the equilibrium distribution. In contrast the glycerol concentration appeared to be the predominant factor determining the proportion between MAG and DAG at equilibrium. This points to a correlation between the glycerol and TAG molar ratio for the outcome of the glycerolysis reaction.

The sequential reaction with MAG as starting material reached a plateau sooner than reactions with DAG which was faster than TAG (Table 7.5). In the DAG system, the MAG formation appeared synchronous to the DAG degradation. In contrast, the TAG system showed a faster DAG formation (equilibrium reached after just 30 min) compared to the 'slower' concurent MAG formation and TAG conversion (equilibrium reached after 70 min). This suggests an ester-exchange from TAG to MAG, concurrent with the ester-exchange from TAG or DAG to glycerol as illustrated in Eq. 4.3 (paragraph 4.1.1). This is in agreement with Moquin *et al.* (2006)

who observed a faster forward reaction rate for the ester-exchange from TAG to MAG compared to the exchange from TAG or DAG to glycerol. This indicates that TAG is converted in the presence of glycerol (Eq. 4.1) as well as MAG (Eq. 4.3). Hence, as the MAG amount increases, the forward MAG formation by a sequential ester-exchange between TAG or DAG and glycerol (Eq. 4.1-4.2) apparently is slowed down by the competitive exchange between TAG and MAG (Eq. 4.3).

As expected, the equilibrium constant for the TAG conversion to DAG and MAG (K_1) exceeded the equilibrium constant for DAG conversion to MAG (K_2) and the reverse reaction (K_3) (Table 7.5). A K_1-value 13 times higher than the K_2-value clearly illustrates that Eq. 4.1 is the overall reaction dominating the glycerolysis system, with very high conversion degrees of the TAG. A K_2-value 25 times higher than the K_3-value implies that MAG formation is favored from DAG formation. The equilibrium constants were validated by comparable reactions conducted over a broader range of glycerol to oil ratios (2-5) in a TB media. Here lower K_1–values were determined (K_1~31 to K_1~41 depending on the glycerol to oil molar ratio tested) while the K_2- and K_3-value were almost identical (K_2~4.3 and K_3-0.2). Although some variations were observed between the two systems, the pattern was believed similar in an overall consideration with a K_1-value superior in number to the K_2-value superior to the K_3-value. This implies that the achieved equilibrium constants seem to be valid at lower glycerol to oil ratios than 5 and in another organic media.

In general, the reaction rates varied considerably depending on reaction media used and compound investigated (Table 7.6).

Table 7.6: Calculated initial reaction rates for the main components obtained from enzymatic glycerolysis in a tert-pentanol system conducted with TAG, DAG and MAG from rape seed oil for 5 min at 50° C.

Initial reaction rate (mol/kg · h)	Reaction medium		
	MAG	DAG	TAG
r_{TAG}	-	-	-0.12
r_{DAG}	0.13	-0.64	0.06
r_{MAG}	-0.52	1.12	0.28
r_{GLY}	0.47	-0.45	-0.43

In the MAG medium, the reaction rate of MAG conversion was observed at a rate similar to the glycerol formation while the rate of DAG conversion was slower.

This suggests undesirable MAG conversion when large amounts of MAGs are present. In the DAG media, the reaction rate for DAG conversion and MAG formation occurred rapid and exceeded all others calculated reaction rates (Table 7.6). This demonstrates that the ester-exchange from DAG to glycerol happened very fast in the presence of high amounts of DAG. In the TAG medium the order of initial reaction rates for glycerol and the acylglycerols were: $r_{GLY} \geq r_{MAG} \geq r_{TAG} \geq r_{DAG}$ (Table 7.6). A DAG formation slower than the TAG conversion indicates that the DAG formation by Eq. 4.1 as well as Eq. 4.3 is the limiting step of the glycerolysis reaction. The relative slow DAG formation in the TAG media compared to the rapid DAG conversion in the DAG media suggests a shift in the reaction rates as the reaction progresses and more MAG and DAG are formed. This shift in reaction rates is supported by similar observations by Moquin *et al.* (2006). The increased amounts of emulsifying MAG/DAG components may be the reason for that. When more glycerol can be emulsified in the oil phase it is likely that the reaction rate is speeded up.

The obtained reaction rates were compared to experiments conducted in a broad spectrum of glycerol to oil ratios in a TB.TP binary mixture (Table 7.7)

Table 7.7: Calculated initial reaction rate after glycerolysis for 30 min. at varied glycerol to oil ratios in a TB:TP media.

Molar ratio Gly: TAG	Initial reaction rate (mol / kg · h)			
	r_{GLY}	r_{TAG}	r_{MAG}	r_{DAG}
0.1	$-1.99 \cdot 10^{-2}$	$-4.51 \cdot 10^{-2}$	$2.36 \cdot 10^{-2}$	$4.20 \cdot 10^{-2}$
1	$-6.83 \cdot 10^{-2}$	$-4.74 \cdot 10^{-2}$	$1.02 \cdot 10^{-1}$	$2.23 \cdot 10^{-2}$
2	$-5.94 \cdot 10^{-2}$	$-4.02 \cdot 10^{-2}$	$8.40 \cdot 10^{-2}$	$1.50 \cdot 10^{-2}$
4	$-5.28 \cdot 10^{-2}$	$-1.63 \cdot 10^{-2}$	$4.32 \cdot 10^{-2}$	$5.56 \cdot 10^{-3}$
6	$1.35 \cdot 10^{-1}$	$-2.28 \cdot 10^{-2}$	$2.90 \cdot 10^{-2}$	$-2.99 \cdot 10^{-3}$
8	$-2.44 \cdot 10^{-2}$	$-1.63 \cdot 10^{-2}$	$2.46 \cdot 10^{-2}$	$8.79 \cdot 10^{-3}$
10	$-2.57 \cdot 10^{-1}$	$1.90 \cdot 10^{-2}$	$2.37 \cdot 10^{-2}$	$-1.22 \cdot 10^{-3}$
20	$-7.93 \cdot 10^{-2}$	$6.07 \cdot 10^{-4}$	$1.65 \cdot 10^{-2}$	$3.75 \cdot 10^{-3}$
50	$-2.68 \cdot 10^{-2}$	$-4.67 \cdot 10^{-3}$	$1.48 \cdot 10^{-2}$	$1.10 \cdot 10^{-3}$
100	$-3.62 \cdot 10^{-2}$	$-2.45 \cdot 10^{-3}$	$1.76 \cdot 10^{-2}$	$-7.09 \cdot 10^{-4}$

Overall, the initial reaction rates of the different components varied at the different glycerol to oil ratio tested but mostly in the same order of magnitude (Table 7.7). Hence, simultaneous reactions most likely occurred concurrently. At most glycerol to oil ratios (except from gly:TAG ratios of 0.1 and 20), the order of initial reaction rates for the acylglycerols and glycerol were: $r_{MAG} / r_{GLY} \geq r_{TAG} \geq r_{DAG}$ with a MAG formation three to 14 times more rapid than DAG formation (Table 7.7). These

findings agree with the findings for the TP system (Table 7.6). It was not possible to detect a tendency towards an altered reaction rate as the glycerol to oil ratio was increased. Hence, no particular effect of the tested glycerol to oil ratios was observed.

7.2.6 External mass transfer limitations

Enzymatic glycerolysis were conducted in two reactors with varied l/d ratio to evaluate plausible external mass transfer limitations (Paper IV). Almost identical time courses with similar MAG formations were observed for the two columns tested (Figure 7.13).

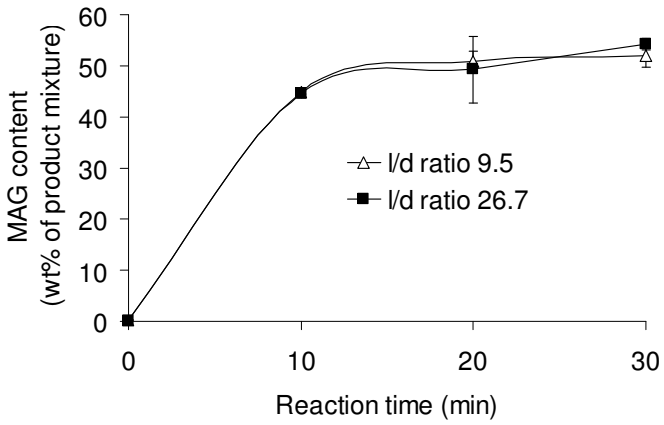


Figure 7.13: Measured MAG content after glycerolysis in columns with varied length-to-diameter ratios but similar reactor volume and identical reaction conditions.

Our results imply that the fluid reactant mixture freely moved past the enzyme particles, independent of the column l/d ratio. To theoretically verify this finding the Sherwood number was calculated (defined in paragraph 5.3). Some differences (13-16%) were observed in the calculated Sherwood number with higher values in the long thin column compared to the shorter thicker column. Although this indicates higher external mass transfer resistance in the long/thin compared to the short/thick column these differences are believed negligible as the differences only varied in the same range as the inaccuracy of the experimental set-up (10-15%). Hence, it seemed unnecessary to reduce the column reactor l/d ratio (≤ 25) to improve the transfer of the fluid mixture through the column.

The effect of different flow rates were evaluated by using different columns dimensions while the reaction time and other conditions were remained unchanged (Table 7.8).

Table 7.8: MAG content and reaction rate obtained from glycerolysis at different flow rates (20 min) in a TB:TP system

Reactor Diameter (mm)	Reactor Length (mm)	Flow (mL/min)	Fluid Velocity m/s	MAG content [a] (wt%)	Reaction rate (r_A)
34	500	11.60	$8.4 \cdot 10^{-5}$	55.6 ± 0.3	7.84
21	500	4.60	$2.2 \cdot 10^{-4}$	52.9 ± 0.4	7.46
21	400	3.50	$1.7 \cdot 10^{-4}$	52.5 ± 0.7	7.40
21	200	1.80	$8.7 \cdot 10^{-5}$	53.9 ± 0.4	7.60
15	200	0.95	$9.0. \cdot 10^{-5}$	50.2 ± 1.5	7.08

[a]Average values of triple determinations ± STD.

Almost identical reaction rates and MAG contents were observed, independent of the different fluid velocities tested (Table 7.8). This indicates that even low fluid velocities are not a critical factor for the process. From the fluid velocities laminar flow was predicted which indicates that the flow dynamics was dominated by viscous forces. This is in good agreement with the high viscous oil and glycerol raw material although the presence of solvent reduced the viscosity. The viscosity of glycerol and oil dissolved in 50 wt% TB:TP mixture (80:20 vol%) was measured to be 62 mPa · s at 25°C. In comparison, pure oil (olive) and glycerol typically have viscosities of ~ 80 and 1500 mPa · s, respectively at room temperature (Lide, 2007b). In a scaled-up reactor with increased dimensions, higher flow rates are used to maintain similar fluid velocity. Thus, it should be kept in mind that higher flow rates can increase the pressure drop leading to practical operating difficulties such as increased risk for pump malfunctions, enzyme bed compression, blockage etc. (Yang *et al.*, 2005b). Anyhow, from the mass transfer findings, a scaled-up continuous glycerolysis process seemed to be feasibly without any great external mass transfer problems.

7.3 MAG purification

7.3.1 Solvent and glycerol separation through membrane filtration

A preliminary screening of membrane filtration for solvent and glycerol removal showed many challenges. The set up was unstable and the outcome of testing

different parameters was not always reproducable. In addition, the lipids components had a tendency to crystallize/clump together during filtration at room temperature. Thus, heating of the product mixture was needed in the membrane cell but resulted in most cases in membrane leakage. In Table 7.9 are the results from the membrane filtration experiments summarized.

Table 7.9: Obtained reaction conditions during membrane ultrafiltration.

Membrane Characteristics				Reaction conditions			
	Selective layer	Support layer	Cut-off (M_w)	Feed inlet	Pressure (bar)	Temperature (°C)	Flux[b] (kg/m^2 · h)
GR81PP	PSf	PP	10,000	Water	3	21	15.8
				Ethanol	3	21	30.9
				TP	3	21	7.9
				Glycerolysis mix in TP[c]	3	21	No flux
ETNA01PP	PVDF[a]		1,000	TP	1.2	60	6.1
					3		17.9
					5		29.8
				Glycerolysis mix in TP	2	60	No flux
					3		4.7
					4		5.0
				Water	3	26	49.3
				Ethanol	3	25	26.6
				TP	3	22	4.9
				TP	3	49	Membrane broke
				Glycerolysis mix in TP	3	46	13.4
				Glycerolysis mix in TP[c]	3	46	No flux
ETNA10A	PVDF[a]	PP	10,000	Water	3	22	26.6
				Ethanol	3	22	23.2
				TP	3	22	4.1
				Glycerolysis mix in TP[c]	3	22	No flux
Cellulose acetate			10,000	TP	2		1.8
					3		2.5
					4		3.6
					5		4.8
				TP+ sunflower oil	5	20	2.7

PSf= polysulphone, PVDF=polyvinylidenefluoride, PP=polypropylene, TP= tert-pentanol
[a]Hydrophilic coated.[b]Calculated as flow /membrane area, with densities set to 0.8 kg/l for TP and 0.85 kg/l for the glycerolysis mix in TP. [c]Initially pre-flushed with water, ethanol and TP

In some trials, membrane pretreatment in water and alcohols were conducted (Table 7.9). This was done to wash out undesirable components from the surface and pores that have been used during the manufacturing process and to promote a stabile

flux before sample filtration. In general, flux of water, ethanol and TP was obtainable while flux from the glycerolysis mix in TP failed to happen. Several attempts were made with increased pressure and temperature to initiate the flux of the glycerolysis mix. However, it only succeeds once at a relative high temperature of 60°C that was inappropriate for the heat sensitive membrane material (Fig. 7.14).

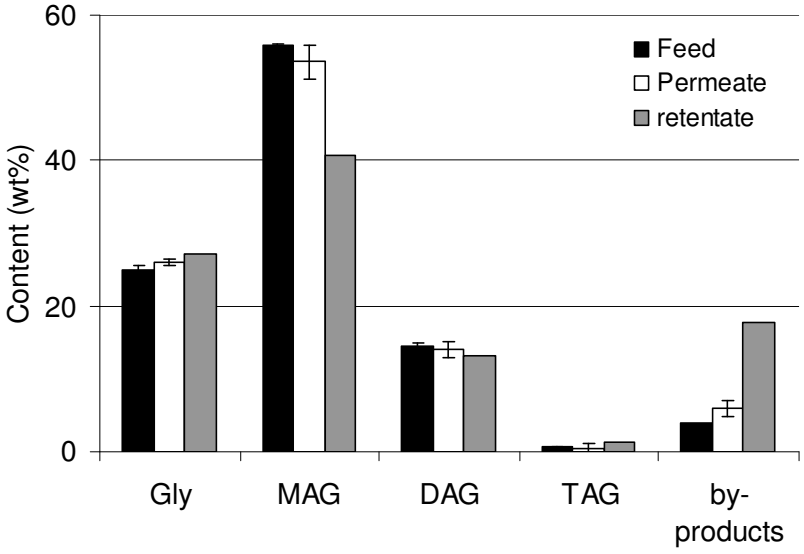


Figure 7.14: Measured compound distribution of MAG, DAG, TAG and glycerol in feed inlet, permeate and retentate (single determination) from a glycerolysis mix in TP filtered through ETNA01PP at 60℃ and 3 bars.

The measured compound distribution of the feed and permeate obtained from the conducted UF were identical (Fig. 7.14). Only minor variations within the inaccuracy of the measurement were observed, confirming the separation failure. A significant lower MAG content was observed in the retentate compared to the feed and permeates samples. This implies that a minor amount of the MAG molecules were separated from the retained mixture. However, retentate contained at the same time a considerable amount of unwanted by-products (FFA and FAE) compared to the feed and retentate. Hence, conversion of the MAGs to these by-products is believed as major reason for a reduced MAG content, rather than an effect of the membrane separation. The reason for this pronounced by- product formation was unknown. A trial with TP performed at 49°C showed a very fast and unstable flux followed by a membrane leakage and deformation of the PVDF-membrane. This confirms that heating of the membrane is not advisable. Hence, usage of membrane filtration as separation technique to purify MAGs from glycerolysis was found doubtful.

7.3.2 Solvent removal by steam stripping

Solvent stripping at a pilot plant facility was successfully conducted without any TP residues obtained in the purified MAG product (Table 7.10).

Table 7.10: Compound distribution before and after solvent stripping at pilot plant facilities at Danisco A/S.

Composition	Feed	After stripping
Tert-pentanol	48.7	0.0
Glycerol	12.4	7.2
Methyl-FFA	0.6	0.7
FFA	0.6	1.3
MAG	26.7	60.2
DAG	10.3	27.7
TAG	0.7	2.9
Total	100	100

These results imply that volatile solvents such as TP and TB can be removed from the glycerolysis mixture by steam stripping. From the external condensator valve, solvent ran out during the solvent stripping. Hence, a better solvent entrapment is required in future experiments, which is believed to be manageable in practice. We did not succeed in removing all the glycerol concurrent with the solvent removal. After stripping, 7 wt% remaining glycerol was observed (Table 7.10). Glycerol amounts up to 10 wt% are current removed from the glycerolysis mixture in chemical MAG production by glycerol stripping, but at higher temperatures of about 190°C and a pressure of 2-5 mbar (Danisco A/S, 2008). Hence, by running the stripping once more with temperatures and pressure adjusted to benefit glycerol evaporation all glycerol can probably be removed. Thus, it is believed reasonably to assume that the product contains only the MAG, DAG and TAG lipids components after the stripping procedure.

7.3.3 MAG separation by shorth path disstillation

With a product expected to consist of only lipid materials after enzymatic glycerolysis and stripping processing, many similarities can be drawn to the anticipated product composition from chemical glycerolysis. The variation lies mainly in the saturation degree of the FA and the chain length and perhaps in the represented acylglycerol distribution. Hence, the use of MAG purification through SPD, similar to

the method current used in chemical processing was believed to be applicable. Besides being a well-known separation technology in the oil industry (Xu, 2005) SPD has the advantages of being a continuous process working under vacuum conditions with low evaporating temperatures and short residence time. This allows distillation of thermosensitive products with minimal stress, all things of great interest for industrial processing of nutritional important MAGs containing PUFAs. Thus, the main concern for using SPD was the separation ability of the unsaturated MAG, DAG and TAGs. In Table 7.11 are the boiling points (b.p) for some unsaturated long chain acylglycerols compared to corresponding saturated acylglycerols.

Table 7.11: Physical properties of solvent, glycerol and some acylglycerols at 101.325 KPa= 1 atm.

Compound	Formula	Melting point (°C)	Boiling point (°C)
Tert-pentanol (TP)	$C_5H_{12}O$	-9.1	102.4
Tert-butanol (TB)	$C_4H_{10}O$	25.7	82.4
Glycerol	$C_3H_8O_3$	18.1	290 130-150[a]
Mono-olein (MAG C-18:1)	$C_{21}H_{40}O_4$	35	405 238-240[b]
Tri-olein (TAG C-18:1)	$C_{57}H_{104}O_6$	-4 [c]	237[c]
Mono-stearin (MAG C-18:0)	$C_{21}H_{42}O_4$	67	407
Di--stearin (DAG C-18:0)	$C_{39}H_{76}O_5$	108	490
Tri-stearin (TAG C-18:0)	$C_{57}H_{110}O_6$	75	552 240[c]

[a] 0.2-0.5 KPa; [b]0.4 KPa; [c] 2.39 KPa.
Ref. Lide, 2007b; Chemfinder, 2008; and Danisco A/S, 2008.

For the long chain FA, the unsaturated and saturated FA had almost identical b.p at comparable pressure (Table 7.11). The monounsaturated mono-olein and tri-olein (C-18:1) had boiling points that only varied 2°C from the corresponding saturated mono-stearin and tri-stearin (C-18:0) (Table 7.11). The three corresponding MAG, DAG and TAG acylglycerols with stearic acids esterified (C-18:0) (Table 7.11), differ sufficiently in b.p (> 60°C) to make SPD possible (Xu, 2005). Based on this, C-18 FA easy obtainable from vegetable oil raw materials, was believed well suited for SPD. Although, the long chained unsaturated FA profile are sensitive to thermal damage the acylglycerols can be feed into the evaporator under low pressure (≤ 0.1 mbar) which reduces the needed operation temperature. Furthermore, the acylglycerols only have a very short distance to the internal positioned condenser in SPD. By this, the evaporation temperature and time is so noticeable decreased that

components are made sufficiently volatile before plausible decomposition and polymization occurs. With these considerations in mind the SPD current applied in industrial plants is believed well suited for the separation of MAG with unsaturated long chain FA as well. Similar to chemical glycerolysis, it is important that all glycerol are removed prior to the SPD. Glycerol has a lower boiling point compared to the acylglycerols, why it easily will be transferred to the distillate of MAGs. Hence, glycerol present in the SPD feed complicates the separation of MAGs.

7.4 Scaled up industrial processing

Findings on laboratory scaled continuous glycerolysis clearly demonstrated a great potential for an industrial process using PBR (Paper IV). To test larger scaled operations this process was transferred to accessible pilot plant equipment having reactor volumes 50 to 400 times bigger compared to the volume used for small scale experiments. In Table 7.12 are the main findings from the scaled up experiments in pilot plants summarized.

Table 7.12: Measured flow rate compared to the pressure drop in pilot plant equipment

Experi-ment	React. Time (τ) min	Flow rate feed inlet kg/h	Fluid velocity m/s	MAG content (wt%)	Pressure drop (bar)	Sherwood number (sh)
PBR-I	10	4.8	$8.63 \cdot 10^{-04}$	44.7± 0.6	25*	3.5
	20	2.4	$4.31 \cdot 10^{-04}$	46.6 ± 2.7	11.5	3.0
	30	1.6	$2.88 \cdot 10^{-04}$	47.0 ± 1.5	8	2.8
PBR-II	10	11.3	$8.63 \cdot 10^{-04}$	42.5 ± 1.7	22	3.5
	20	5.6	$4.31 \cdot 10^{-04}$	49.0 ± 0.6	11	3.0
	30	3.8	$2.88 \cdot 10^{-04}$	51.1 ± 0.3	7	2.8
FILTER-I	10	11.3	$4.97 \cdot 10^{-05}$	41.6 ± 5.6	0	2.3
	20	5.6	$2.48 \cdot 10^{-05}$	50.8 ± 1.3	0	2.2
	30	3.8	$1.66 \cdot 10^{-05}$	53.6 ± 0.7	0	2.2

**Experiment was stopped due to high pressure drop.*

All three tested set ups resulted in high MAG yields from 45 wt% to 54 wt% in short time (Table 7.12) similar to the results from comparable small scale experiments (Paper IV). At 30 minutes, the achieved MAG contents from 47 wt% to 54 wt% were all close to achievable equilibrium MAG amounts of 50-55 wt% (Paper II). However, after 30 minutes of reaction, the filter based set-up provided a significant higher MAG content compared to the contents obtained from the PBR columns (Table 7.12). Thus, the filter based set-up obviously improved the contact

between enzyme and reactant mixture, indicating better mass transfer. This, although no distinct mass transfer limitations were seen in the corresponding small scale experiments having similar column l/d ratios and flow rates (Paper IV). The significant lower Sherwood number calculated for the filter based set-up compared to the PBR set-ups (Table 7.12) theoretically verify better mass transfer in the filter based set-up. The flow rates were adjusted to obtain similar residence times in the fixed enzyme layers, resulting in a more slow running feed inlet in the PBR-I experiments compared to the PBR-II and FILTER-I flow rates (Table 6.8, paragraph 6.4). Although reduced flow rates increased the risk of external mass transfer limitation the used fluid velocity were similar to small scale experiments. Hence, the indicated PBR mass transfer limitations might be controlled by the enzyme bed dimensions rather than the flow rates. PBR I and PBR II had l/d ratios of ~21.2 and ~13.9, respectively while the filter based set up had an enzyme bed l/d ratio of just ~0.2. To awoid the high pressure drops from the PBR set-ups a l/d ratios less than approximately ~10 is believed most beneficial in future experiements.

The equipment facilities were set up and packed with enzyme just once. In each set up, three different reaction times were therefore tested by starting with the slow flow rate (corresponding to a reaction time of 30 min) and increased as the reaction times were shortened. Hence, PBR experiments having the greatest pressure drops (shortest reaction time) were performed last. This indicates a compressed and a plugged enzyme bed as operation progressed. The applied down flow in combination with a very small column diameter is believed to be the reason for this.

Although the down flow apparently compressed the PBR enzyme beds it was believed well suited for the filter based column to provide a better entrapment of the enzyme bed. The filter set up had a large dead volume after enzyme filling and wetting and a much greater cross section area compared to the PBRs (Table 7.12). Thus, an up flow may open channels through the wide and shallow enzyme bed. Hereby, the capability of some of the reactants to bypass the enzyme with a corresponding reduction in the efficiency is very likely. An up flow might carry out enzyme particles from the enzyme layer, although a filter plate added next to the outlet should avoid this.

Based on the findings the wide filter based column with a shallow enzyme bed seemed to be the best choice for future enzymatic industrial applied glycerolysis. The tested filter equipment was small compared to typically industrial plants. Even this 'small' sized set-up clearly illustrated a high capacity. Based on an enzyme life time of 2200 h enzyme, found in the small scale PBR experiments (Paper IV), and a reactant flow of 3.75 kg/h, 25 wt% of the product mixture ending up being purified MAGs, and a enzyme consumption of barely 0.9 kg, approximately 2.0 tons purified MAGs were predicted from the actual process. Based on this prediction the enzyme cost per kg pure MAG was estimated to 0.5 EUR (Table 7.13).

Table 7.13: Estimated raw material cost for production of pure MAGs from enzymatic glycerolysis based on 50wt%TB:TP 80:20 vol% solvent and a glycerol to oil ratio of four.

Raw material	Price EUR/kg*	Amounts (kg)	Cost per kg MAG* (EUR)
Glycerol	1.1	1184	0.65
Rape seed oil	0.95	2816	1.34
98% pure TP	6.5	800	2.60
99% pure TB	5.4	3200	8.64
Novozym®435	1060	0.9	0.48
Pure MAG		2000	13.71

**Based on industrial prices obtained from Danisco A/S, 2008.*

With all raw materials included, the cost price is nearly 14 EUR per kg MAG. However, re-use of solvents and non reacted glycerol dramatically diminishes the cost, estimated to be approximately 2 EUR per kg pure MAG with enzyme, oil and partly glycerol being included (Table 7.13). Although production costs (energy consumption, facilities etc.) are excluded from the cost-estimations, the re-use of solvents and non reacted glycerol makes raw material costs of 2 EUR for 'health improved' pure MAG products reasonable. Thus enzymatic production of MAG is believed realistic from economical point of view. However, the 'high' costs' underline the importance of special applications due to more cost competitive MAGs from the highly efficient chemical glycerolysis.

CONCLUSIONS AND FUTURE OUTLOOK

8. Overall conclusions and perspective of the project

The development of an industrial applicable enzymatic process to obtain highly pure MAG products from vegetable oils containing long chained PUFA was accomplished during the Ph.D work (Fig 8.1).

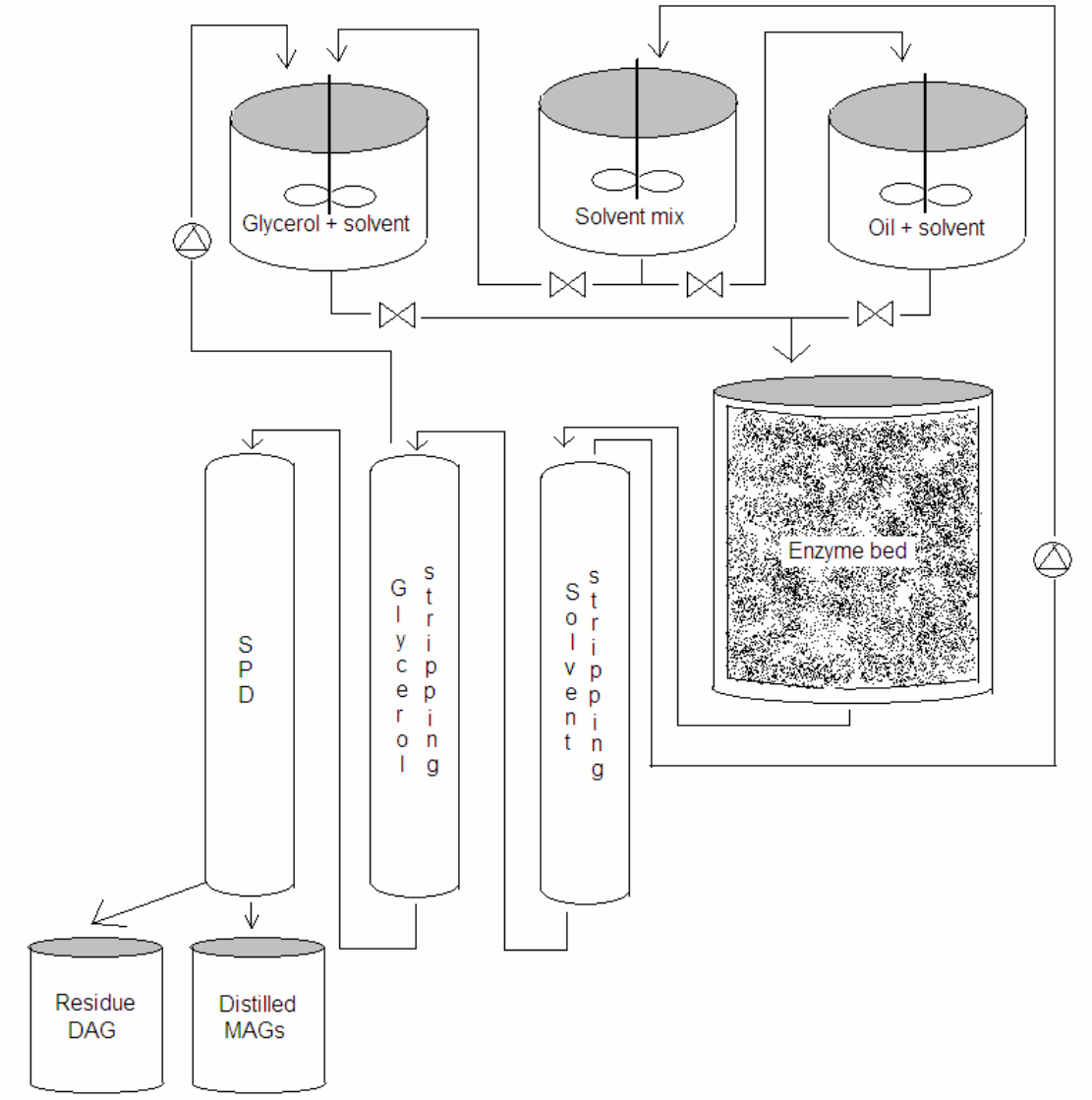


Figure 8.1: Illustration of an suggested enzyme-catalyzed industrial applicable process for partial acylglycerol production

The main conclusions from evaluation of varied parameters and experience from first pilot plant trials are summarized in the following:

Preparation of raw material:

- A used glycerol to oil molar ratio of 2 is recommended to obtain a low glycerol and TAG amount (of approximately 8 and 9wt%, respectively) and relatively high content

of MAG and DAG (44 and 39 wt%, respectively). This ratio is believed most beneficial for production of MAG + DAG mixtures.
- A glycerol to oil molar ratio of 4-5 is recommended to obtain maximal MAG content of 55 wt%, and a high conversion degree of the TAG (97%) although DAG and glycerol (16 wt% and 20 wt%, respectively) still are present after completed reaction. This ratio is believed most beneficial to produce highly purified MAG products.
- In the reaction mixture solvent addition, preheating to minimum 40°C and continuous stirring is needed to ensure a sufficient dispersion of glycerol and oil and a 'low' reactant viscosity.
- The binary TB: TP media (80:20 vol%), in amount of 44-50wt% is found most beneficial for use in the glycerolysis reaction with respect to greatly enhanced reaction efficency, practical handling and resonable costs.

MAG synthesis in PBR
- Novozym®435 can be used as supplied from manufacturer poured directly into a column reactor in its dry form.
- Recommended enzyme filling amount is approximately 220-230 kg/m^3 column.
- Initial wetting of enzyme bed and stabilization of column is required for approximately 1-2 hours with reactant mixture.
- The life time of Novozym®435 is estimated at 2200 h and a capacity of 2 tons pure MAG per kg enzyme used
- A glycerolysis reaction temperature of 40°C is found appropiate
- l/d ratio < 10 and fluid velocities in range from $1.7 \cdot 10^{-5}$ to $5.0 \cdot 10^{-5}$ m/s seems suitable to avoid mass transfer limitations and minimize pressure drops
- A reaction time of minimum 20 minutes is found to be optimal to efficient reaction reaching equilibrium conditions.
- The reaction rate of TAG conversion to DAG seems to be the limiting step for MAG formation.
- Only a minor amount of undesirable FFA and FAE (a few weight percentages) were formed during reaction ascribed a combination of impurities such as 2-butanol and saturated FA present in the system.

MAG purification:
- Re-use of glycerol and solvent is required after removal by steam stripping to lower the raw material costs and to minimize waste.
- SPD is believed capable of separating heat senstive acylglycerols carrying important unsaturated PUFAs to obtain highly pure MAG (≥ 95%).
- The sn-ratio of the formed MAGs is believed controlled by thermodynamically favorable acyl-migration, ending up with approximately 90:10 sn-1 (3):sn-2 MAGS.

In an overall perspective, the developed process was concluded very suited to access the large scaled industrial enzymatic glycerolysis due to simple operations, high reaction efficiency and obtainable health improved MAG products. In addition, the process benefits from the know-how related to purification processing already used in the chemical glycerolysis. Although, further up-scale experiments is needed before a large scale production can be started our results provide a well documented basis. The questions which remains unanswered from the present study relates in

particular to practical handling challenges that we believe can be solved by further up-scale design improvements, process engineering etc. The process development has been the major focus area in the present study rather than the product quality. Hence, little attention have been paid to the quality of the produced MAGs. Even so, we believe that the assistance of a nitrogen flow can minimize undesirable oxidation of the sensitive PUFAs to obtain high MAG quality. However, in the future, a detailed quality evaluation of the produced MAGs containing sensitive PUFAs is required to confirm that the processing leaves the sensitive PUFAs undamaged.

By implementing an enzyme catalyzed method to produce MAGs from unsaturated raw materials at ambient temperatures an industrial 'white technology' can be introduced. In recent years, companies have become more and more aware of taking corporate responsibility and utilize sustainable production methods. Hence, using 'white biotechnolgy' is believed as the future way to produce MAGs. Although using solvents is conflicting with a safe and healthy production method and food applications, avoiding solvents is difficult. To maintain efficient contact between the lipophillic oil and the hydrophilic glycerol and lipase enzyme at ambient temperatures some kind of 'dilution medium' is required. Anyhow, as long as safety precautions are taken during handling of the tertiary alcohols, these solvents are not considered to possess any special harmful risk. Hence, industry needs to accept the solvent in the first generation industrial applied enzymatic glycerolysis until an alternative is found.

The enzymatic glycerolysis was not found cost competetive to the chemical glycerolysis due to extensively increased solvent and catalyst costs. Hence, replacing the chemical synthesis with the more costly enzymatic synthesis for MAG products serving as emulsifiers in non-food and 'low valued' applications is unrealistic. However, the enzymatic process has the advantages over the chemical process by its valuable capability to produce nutritional enhanced MAG components. The vegetable oils which are used for the MAG synthesis have to compete with many other applications including non edible purposes such as raw material for biodiesel production etc. However, there will always be a demand for food ingredients and health enriched compounds. Hence, the enzyme catalysed method can lead the way in making healthier emulsifiers and make a difference for MAGs future use in food, pharmaceutical, cosmeceutical and nutraceutical.

With an continuous increasingly consumer awareness on not only basic nutritional benefits but also disease prevention and health enhancing compounds in the diet, the market interest is definitely present. The succesful story of the commercial produced health improved DAG cooking oil rich in unsaturated fatty acids (Enova™oil) clearly illustrates that consumers can relate to dietary fat with healthy characteristics and are ready for commercialisation of health improved fats (and deviates). Hence, MAGs containing essential PUFAs are believed realistic ingredients in dietary supplements or as addition to foods in the coming years. Whether implementation of these products are profitable relies on the balance between the demands for healthier food products and the production cost and therefore ultimately the cost for consumers.

Based on 20 years of progressing research in the field of lipid- and lipase technolgy, the enzymatic glycerolysis for partial acylglycerol production is believed well documented and ready from a scientific point of view. The first global companies have succesfully implemented lipase catalyzed esterification/ interesterification processing into commercial plants to launch healt improved lipid products. This has made the lipase catalyzed bioprocessing more well established and the industry now seems to slowly accept further utilization of such 'bioprocesses'. The tremendous development in oil and fat industry in recent years clearly confirms the ongoing industrial trend away from traditional thinking. From its sole focus on traditional margarine dominated by saturated fat and oils, the industry has now turned towards low-fat spreads, trans-free solutions and structured lipids with specific FA (Akoh, 2005). Hence, the fat and oil industry is now facing that lipase catalyzed processing is very useful/helpful to obtain such health improved fats and fats deviates. Although, the enzyme cost are relatively high, efficient, immobilized lipases are availible on the global biocatalysts market to access a uniform and consistent enzyme supply to large scale plants. Hence, time has come to pass for industry to adopt bioprocesses for nutritiuous MAG production. The next step is therefore for potential global MAG suppliers to invest in an up-scaled enzymatic glycerolysis process. If these investments are successful the potential gained will be huge.

REFERENCES

Aha, B.; Berger, M.; Hermann, J.; Keil, O.; Waldinger, C.; Schneider, M. **(1998).** Food emulsifiers: Enzyme assisted synthesis of isomerically pure mono- and diglycerides. *Food Sci. Technol. Today*, 12 (1), 30-34.

Akoh, C. C. **(1993).** Lipase-Catalyzed Synthesis of Partial Glycerides. *Biotechnol. Lett.* 15 (9), 949-954.

Akoh, C. C. **(2005).** Structured and Specialty Lipids. In *Healthful Lipids*, eds. Akoh, C. C.; Lai, O-M. AOCS Press, Champaign, Illinois, USA, 591-606.

AOCS **(1997).** Determination of Mono- and Diglycerides by Capillary Gas Chromatography. AOCS Official Method Cd 11b-91, Ed. American Oil Chemists' Society, 1-5.

Arcos, J. A.; Garcia, H. S.; Hill, C. G. **(2000).** Continuous enzymatic esterification of glycerol with (poly)unsaturated fatty acids in a packed-bed reactor. *Biotech. Bioeng.* 68 (5), 563-570.

Arcos, J. A.; Hill Jr.; C. G; Otero, C. **(2001).** Kinetics of the Lipase-Catalyzed Synthesis of Glucose Esters in Acetone. *Biotech. Bioeng.* 73 (2), 104-110.

Barros, R. J.; Wehtje, E.; Adlercreutz, P. **(1998).** Mass Transfer Studies on Immobilized a -Chrymotrypsin Biocatalysts Prepared by Deposition for Use in Organic Medium. *Biotech. Bioeng.* 59 (3), 364-373.

Bellot, J. C.; Choisnard, L.; Castillo, E.; Marty, A. **(2001).** Combining solvent engineering and thermodynamic modeling to enhance selectivity during monoglyceride synthesis by lipase-catalyzed esterification. *Enzyme Microb. Technol.* 28 (4-5), 362-369.

Bornscheuer, U.; Stamatis,H.; Xenakis, A.; Yamane,T.; Kolisis, F. N. **(1994).** A comparison of different strategies for lipase-catalyzed synthesis of partial glycerides. *Biotechnol. Lett.* 16 (7), 697-702.

Bornscheuer, U. T. **(1995).** Lipase-catalyzed synthesis of monoacylglycerols. *Enzyme Microb. Technol.* 17 (7), 578-586.

Bousquet-Duboch, M-P.; Graber, M.; Sousa, N.; Lamare, S.; and Legoy, M-D **(2001).** Alcoholysis catalyzed by Candida Antarctica lipase B in a gas/solid system obeys a Ping Pong Bi Bi mechanism with competitive inhibition by the alcohol substrate and water. *Biochem. Biophys. Acta* 1550, 90-99.

Boyle, E.; German, J. B. **(1996).** Monoglycerides in Membrane Systems. *Crit. Rev. Food Sci. Nutr.* 36 (8), 785-805.

Boyle, E. **(1997).** Monoglycerides in Food Systems: Current and Future Uses. *Food Technol.* 51 (8), 52-59.

Chang, P. S.; Rhee, J. S.; Kim. J. J. **(1991).** Continuous Glycerolysis of Olive Oil by Chromobacterium-Viscosum Lipase Immobilized on Liposome in Reversed Micelles. *Biotech. Bioeng.* 38 (10), 1159-1165.

Cheirsilp, B.; Kaewthong, W.; H-Kittikun, A. **(2007).** Kinetic study of glycerolysis of palm olein for monoacylglycerol production by immobilized lipase. *Biochem. Eng. J.* 35, 71-80.

Chemfinder **(2008).** Homepage: www.chemfinder.com accessed 13.02.2008.

Coteron, A.; Martinez, M.; Aracil, J. **(1998)** Reactions of Olive Oil and Glycerol over Immobilized Lipases, *J. Am. Oil Chem. Soc.* 75, 657-660.

Danisco A/S **(1989).** Monoglyceride Analysis by Capillary Gas Chromatography. Technical Paper TP 1-1e. Ed. Jensen, J. D., 1-7.

Danisco A/S **(2008).** Homepage: www.danisco.com accessed 14.04.2008 and oral communication.

Danisco Cultor **(2000).** Analytical Manual A0334.

Danisco Ingredients **(1998/99).** GC-Forskrift GC Nr. 1 & GC Nr. 2.

deMoura, J. M. L. N.; Goncalves, L. A. G.; Sarmento, L. A. V.; Petrus, J. C. C. **(2007).** Purification of structured lipids using $SCCO_2$ and membrane process. *J. Membr. Sci.* 299, 138-145.

EFEMA **(2004).** E471: Mono- and diglycerides of fatty acids. In *EFEMA Index of Food Emulsifiers.* Ed. European Food Emulsifier Manufactures Association, 4th ed., 51-54. Accessed from www.emulsifiers.org 04.04.2005.

Elfmann-Börjesson, I.; Härröd, M. **(1999).** Synthesis of Monoglycerides by Glycerolysis of Rape seed Oil Using Immobilized Lipase, *J. Am. Oil Chem. Soc*. 76, 701-707.

Enovaoil **(2008).** Homepage: www.enovaoil.com accessed 13.05.2008.

Fadiloglu, S.; Ciftci, O. N.; Gögüs, F. **(2003).** Reduction of free Fatty Acid Content of Oliver-pomace Oil by Enzymatic Glycerolysis. *Food Sci. Tech. Int.* 9 (1), 11-15.

Ferreira-Dias, S.; da Fonseca, M. M. R **(1995).** Glycerolysis of olive oil: Batch operational stability of *Candida rugosa* lipase immobilized in hydrophilic polyurethane foams. *Bioprocess Eng.* 13, 311-315.

Ferreira-Dias, S.; Correia, A. C.; Baptista, F. O.; da Fonseca, M. M. R. **(2001).** Contribution of response surface design to the development of glycerolysis systems catalyzed by commercial immobilized lipases. *J. Mol. Catal. B-Enz.* 11 (4-6), 699-711.

Flickinger, B. D.; Matsou, N. **(2003).** Nutritional Characteristics of DAG oil. *Lipids*, 38, 129-132.

FNB. **(2004).** General tests and assays. In *Food Chemical Codex*, ed. Food and Nutrition Board, Institute of Medicine of the National Academies. National Academy Press, Washington DC, 5th edn. 827-962.

Freedman, B;. Pryde, E. H.; Kwolek. W. F. **(1984).** Thin-Layer Chromatography Flame Ionization Analysis of Transesterified Vegetable-Oils. *J. Am. Oil Chem. Soc.* 61 (7), 1215-1220.

Fukunaga, K.; Mine, Y.; Samejima, K-I.; Yoshimoto, M.; Nakao, K.; Sugimura, Y. **(2002).** Enhancement of *Candida rugosa* lipase activity in non-aqueous media by co-lyophilization with amphiphiles from aqueous solution. *Biotechnol. Lett.* 24, 1157-1160.

Fureby, A. M **(1995).** Lipases. In *Aspects on lipase-catalysed preparation of partial glycerides.* Akademisk Avhandling, Department of Biotechnology, Lund, Sweden 25-32.

Garcia, H. S.; Yang, B.; Parkin, K. L. **(1996).** Continuous reactor for enzymatic glycerolysis of butter oil in the absence of solvent. *Food Res. Int.* 28, 605-609.

Garcia, H. S.; Arcos, J. A.; Keough, K. J.; Hill, C. G. **(2001).** Immobilized lipase-mediated acidolysis of butter oil with conjugated linoleic acid: batch reactor and packed bed reactor studies. *Mol. Catal. B-Enz.*11 (4-6), 623-632.

Gebauer, S.; Harris, W. S.; Kris-Etherton, P. M.; Etherton, T. D. **(2005).** Dietary n-6:n-3 Fatty Acid Ratio and Health. In *Healthful Lipids*, eds. Akoh, C. C.; Lai, O-M. AOCS Press, Champaign, Illinois, USA, 221-248.

Goto, M.; Kamiya, N.; Nakashio, F. **(1995).** Enzymatic Interesterification of Triglyceride with Surfactant-Coated Lipase in Organic Media. *Biotech. Bioeng.* 45 (1), 27-32.

Goto, M.; Hatanaka, C.; Goto, M. **(2005).** Immobilization of surfactant-lipase complexes and their high heat resistance in organic media. *Biochem. Eng. J.* 24, 91-94.

Govindaswamy, S.; Andiappan, A.; Lakshmanan, S. **(1977).** Vapour-liquid equilibriums of the binary and ternary systems containg *n*-hexane (1)-benzene (2)-*tert*-butyl alcohol (3) at 760 mmHg pressure. *J. Chem. Eng. Data.* 22, 264-269.

Graber, M.; Irague, R.; Rosenfeld, E.; Lamare, S.;Franson, L.; Hult, K. **(2007).** Solvent as inhibitor for *Candida Antarctica* lipase B. *Biochem. Biophys. Acta.* 1774 (8), 1052-1057.

Grochulski, P.; Li, Y.; Schrag, J. D.; Bouthillier, F.; Smith, P.; Harrison, D.; Rubin, B.; Cygler, M. **(1993).** Insights into Interfacial Activation from an Open Structure of *Candida rugosa* Lipase. *J. Biol. Chem.* 286 (17), 12843-12847.

Gulati, R.; Arya, P.; Malhotra, B.; Prasad, A.K.; Saxena, R.K.; Kumar, J.; Watterson, A. C.; Parmar, V.S. **(2003).** Novel Biocatalytic Esterification Reactions on Fatty Acids: Synthesis of Sorbitol 1(6)-Monoesterate, *Arkivoc* 200, 159-170.

Hahn, H.-D.; Dämbkes, G.; Rupprich, N. **(2008).** Butanols. In *Ullmann's Encyclopedia of Industrial Chemistry.* Wiley-VCH, Weinheim, Germany. 7th ed.

Hansen, H. K.; Rasmussen, P.; Fredenslund, A.; Schiller, M.; Gmehling, J. **(1991).** Vapor-Liquid Equilibria by UNIFAC Group Contribution. 5. Revision and Extension. *Ind. Eng. Chem. Res.* 30, 2352-2355.

Hayes, D. G. **(2000).** Lipid Modification in Water-in-Oil Microemulsion. In *Enzymes in Lipid Modification*, ed. Bornscheuer, U. T., Wiley-VCH, Weinheim, Germany, 46-84.

Hernández-Martín, E.; Otero, C. **(2008).** Different enzyme requirements for the synthesis of biodiesel: Novozym®435 and Lipozyme®TL IM. *Biosource Technol.* 99, 277-286.

Hess, R.; Bornscheuer, U.; Capewell, A.; Scheper, T. **(1995).** Lipase-Catalyzed Synthesis of Monostearoylglycerol in Organic-Solvents from 1,2-O-Isopropylidene Glycerol. *Enzyme Microb. Technol.* 17 (8), 725-728.

Hornstra, G. **(2004).** Omega-3 long chain polyunsaturated fatty acids and health benefits In *Amended and updated version of the english translation of Omega-3 et bénéfice santé.* Ed. Anselmino, C. NutriScience.

http://www-bioeng.ucsd.edu/~herrgard/activation.html. Accessed 04.06.2008.

Iatroscan **(2001).** TLC/FID Instrument Application Appendix-II No. 5: Analysis of Lipids by the Iatroscan In *Instruction Manual for Iatroscan MK-6.*

Irimescu, R.; Furihata, K.; Hata, K.; Yamane, T. **(2003).** Patent EP1300470A1: Process for the production of glycerides with lipases.

Ison, A. P.; Macrae, A. R.; Smith, C. G.; Bosley, B**. (1994).** Mass Transfer Effects in Solvent-Free Fat Inetresterification Reactions: Influences on Catalyst Design. *Biotech. Bioeng.* 43, 122-130.

Iupac **(2008).** Iupac organizations homepage: http://old.iupac.org/reports/2001/colloid_2001/manual_of_s_and_t/node33.html. Accessed 16.05.2008

Jackson, M. A.; King, J. W. **(1997).** Lipase-catalyzed glycerolysis of soybean oil in supercritical carbon dioxide. *J. Am. Oil Chem. Soc.* 74 (2), 103-106.

Jaeger, K.-E.; Eggert, T. **(2002).** Lipases for biotechnology. *Biotechnol.* 13, 390-397.

John, V. T.; Abraham G. **(1991).** Lipase catalysis and its applications. In *Biocatalysts for Industry*, ed. Dordick, J. S. Plenum Press, New York, 193-218.

Johnson, L. A. **(1998).** Recovery, refining, converting, and Stabilizing Edible Fats and Oils. In *Food Lipids.* Eds. Akoh, C. C.; Min, D. B. Marcel Dekker USA, 181-228.

Kaewthong, W.; H-Kittikun, A. **(2004).** Glycerolysis of Palm Olein by Immobilized Lipase PS in Organic Solvents, *Enzyme Microb. Technol.* 35*,* 218-222.

Kaewthong, W., Sirisansaneeyakul, S., Prasertsan, P., & H-Kittikun, A. **(2005).** Continouous production of monoacylglycerols by glycerolysis of palm oil with immobilized lipase, *Process Biochem.* 40, 1525-1530.

Kaneda I,; Vincent B. **(2004).** Swelling behavior of PMMA-g-PEO microgel particles by organic solvents. *J. Colloid Interf. Sci.* 274, 49-54.

KBA **(2007).** Kemibrugs homepage www.kemibrug.dk. Accessed 07.12.2007

Klibanov, A. M. **(2001).** Improving enzymes by using them in organic solvents. *Nature* 409, 241-246.

Kristensen, J. B.; Xu, X., Mu, H. **(2005).** Diacylglycerol Synthesis by Enzymatic Glycerolysis: Screening of Commercially Availble Lipases. *J. Am. Oil Chem. Soc.* 82 (5), 329-334.

Krog, N **(1979).** Dynamic and Unique Monoglycerides. *Cereal Food World*. 24 (1), 10-11.

Krog, N. **(1997).** Food Emulsifiers. In *Lipid Technologies and Applications,* ed. Gunstone, F.D., Marcel Dekker, New York, 521-534.

Kumar, N. S. K.; Bhowmick, D. N. **(1996).** Separation of fatty acids/triacylglycerols by membranes. *J. Am. Oil Chem. Soc, 73 (3), 399-401.*

Kwon, S. J.; Han, J. J.; Rhee, J. S. **(1995).** Production and In-Situ Separation of Monoacylglycerol Or Diacylglycerol Catalyzed by Lipases in *n*-Hexane. *Enzyme Microb. Technol.* 17 (8), 700-704.

Lappe, P.; Hofmann, T. **(2008).** Pentanols In *Ullmann's Encyclopedia of Industrial Chemistry.* Wiley-VCH, Weinheim, Germany. 7th ed.

Laszlo, J. A.; Compton, D. L.; Vermillion, K. E. **(2008).** Acyl migration kinetics of Vegetable Oil 1,2-Diacylglycerols. *J. Am. Chem. Soc*. 85, 307-312.

Lee, G.-C.; Wang. D.- L.; Ho,Y.-F.; Shaw, J.-F. **(2004).** Lipase-Catalyzed Alcoholysis of Triglycerides for Short-Chain Monoglyceride Production, *J. Am. Oil Chem. Soc.* 81(6), 533-536.

Levenspiel, O. **(1999).** Solid Catalyzed Reactions. In *Chemical Reaction Engineering*. Ed. Levenspiel, O. John Wiley & Sons, New York, USA, 376-426.

Li, D.; Bode, O.; Drummond, H.; Sinclair, A. J. **(2003).** Omega-3 (*n*-3) fatty acids. In *Lipids for Functional Foods and Nutraceuticals.* Ed. Gunstone, F.D. The Oily Press, Bridgewater, England, 225-262.

Lide, D. R. **(2007a-2008).** Octanol-Water Partition Coefficients. In *Handbook of Chemistry and Physics,* ed. Lide D.R, CRC Press, 87th edition. [on-line] section 16

Lide, D. R. **(2007b-2008).** Physical Constants of Organic Compounds, In *Handbook of Chemistry and Physics.* Ed. Lide, D.R., CRC Press, 87th edition. [on-line] section 3

Lide, D. R. **(2007c-2008).** Definition of scientific terms, In *Handbook of Chemistry and Physics.* Ed. Lide D.R, CRC Press, 87th edition. [on-line] section 2

Malcata, F. X.; Reyes, H. R.; Garcia, H. S.; Hill, C. G.; Amundson, C. H. **(1992).** Kinetics and Mechanisms of Reactions Catalyzed by Immobilized Lipases. *Enzyme Microb. Technol.* 14 (6), 426-446.

Manjula, S.; Subramani, R. **(2006).** Membrane Technology in Degumming, Dewaxing, Deacidifying, and Decolorizing Edible Oils. *Crit. Rev. Food Sci. Nutr. 46 (7), 569-592.*

Martinelle, M.; Holmquist, M.; Hult, K. **(1995).** On the interfacial activation of *Candida antartica* lipase A and B as compared with *Humicola lanuginose* lipase. *Biochem. Biophys. Acta.* 1258 (3), 272-276.

Martinelle, M.; Hult, K. **(1995).** Kinetics of acyl transfer reactions in organic media catalysed by *Candida antartica* lipase B. *Biochem. Biophys. Acta.* 1251 (2), 191-197.

Matsou, N. **(2005).** Nutritional Characteristics of Diacylglycerol Oil and Its Health Benefits. In *Healthful Lipids*, eds. Akoh, C. C.; Lai, O-M. AOCS Press, Champaign, Illinois, USA, 685-698.

McClements, D. J. **(1998).** Lipid-Based Emulsions and Emulsifiers. In *Food Lipids.* Eds. Akoh, C. C.; Min, D. B. Marcel Dekker USA, 55-88.

McNeill, G. P.; Shimizu, S.; Yamane, T. **(1990).** Solid-Phase Enzymatic Glycerolysis of Beef Tallow Resulting in A High-Yield of Monoglyceride. *J. Am. Oil Chem. Soc.* 67 (11), 779-783.

McNeill, G. P.; Yamane, T. **(1991).** Further Improvements in the Yield of Monoglycerides During Enzymatic Glycerolysis of Fats and Oils. *J. Am. Oil Chem. Soc.* 68 (1), 6-10.

McNeill, G. P.; Yamane, T. W.; Shimizu, S. **(1991).** Patent EP0445692A3: A method of producing monoglyceride.

McNeill, G. P.; Berger, R. G. **(1993).** Enzymatic Glycerolysis of Palm Oil Fractions and a Palm Oil Based Model Mixture - Relationship Between Fatty-Acid Composition and Monoglyceride Yield. *Food Biotechnol.* 7, 75-87.

Millqvist, F. A.; Vitro, C.; Adlercreutz, P.; & Mattiasson, B. **(1996).** Acyl group migrations in 2-monoolein. *Biocatal. Biotransform.* 14 (2), 89-111.

Monteiro, J. B.; Nascimento, M. G., Ninow, J. L. **(2003).** Lipase-Catalyzed Synthesis of Monoacylglycerol in a Homogenous system. *Biotechnol. Lett.* 25, 641-644.

Montgomery, D. C. **(1997).** Response Surface Methods and Other Approaches to Process Optimization, In *Design and Analysis of Experiments.* Ed. Montgomery, D. C., John Wiley & Sons, New York, 4th edn., 427-500.

Moonen, H.; Bas, H**. (2004).** Mono- and Diglycerides, In *Emulsifiers in Food Technology.* Ed. Whitehurst, R. J. Blackwell Publishing Ltd, Oxford, 40-58.

Moquin, P. H. L.; Temelli, F.; King, J. W.; Palcic, M. M. **(2005).** Kinetic modeling of the Glycerolysis Reaction for Soybean Oils in Supercritical Carbon Dioxide Media. *J. Am. Chem. Soc.* 82 (8), 613-617.

Moquin, P. H. L.; Temelli, F.; Sovová, H; Saldaña, M. D. A. **(2006).** Kinetic modeling of the glycerolysis-hydrolysis of canola oil in Supercritical Carbon Dioxide Media using equilibrium data. J. *Supercrit. Fluids,* 37, 417-424.

Moreno, P. A. G.; Medina, A. R.; Rubio, F. C.; Paéz, B. C.; Cerdan, L. E.; Grima, E. M. **(2005).** Production of Structured Triacylglycerols in an immobilised lipase packed-bed reactor: batch mode operation, *J. Chem. Technol. Biotechnol.* 80, 35-43.

Mu, H.; Høy, C.-E. **(2004).** The digestion of dietary triacylglycerols. *Prog. Lipid Res*. 43, 105-133.

Myrnes, B.; Barstad, H.; Olsen, R. L.; Elvevoll, E .O. **(1995).** Solvent-Free Enzymatic Glycerolysis of Marine Oils, *J. Am. Oil Chem. Soc.* 72, 1339-1344.

Murty, V. R.; Bhat, J.; Muniswaran, K. P. A. **(2004).** Mass transfer in Immobilized Lipase Packed Bed Reactor during the Hydrolysis of Rice Bran Oil. *Chem. Biochem. Eng. Q.* 18 (2), 177-182.

Murty, V. R. C.; Bhat, J.; Muniswaran, K. P. A. **(2005).** External Mass transfer Effects during the Hydrolysis of Rice Bran Oil in Immobilized Lipase Packed Bed Reactor. *Chem. Biochem. Eng. Q.* 19 (1), 57-61.

Nakaoki, T.; Mei, Y.; Miller, L. M.; Kumar, A.; Kalra, B.; Miller, M. E.; Kirk, O.; Christensen, M.; Gross, R. A. **(2005).** *Candida antartica* lipase B catalyzed polymerization of lactones: Effects of immobilization matrices on polymerization kinetics & molecular weight. *Ind. Biotechnol.* 1 (2), 126-134.

Nishiba, Y.; Sato, T.; Suda, I. **(2000).** Convenient method to determine free fatty acid of rice using thin-layer chromatography and flame-ionization detection system. *Cereal Chem.* 77 (2), 223-229.

Noureddini, H., Harmeier, S. E. **(1998).** Enzymatic Glycerolysis of Soybean Oil. *J. Am. Chem. Soc.* 75 (10), 1359-1365.

Novozymes A/S **(2001).** Product Sheet for Novozym435, Oils & Fats/ 2001-06950-03.pdf. Novozymes A/S, Bagsværd, Denmark, 1-3.

O'Keefe, S. F **(1998).** Nomenclature and Classification of Lipids. In *Food Lipids.* Eds. Akoh, C. C.; Min, D. B. Marcel Dekker USA, 1-36.

OOPEC **(2004).** CONSLEG: 1995L0002-29/01/2004. European Parliment and Council Directive No. 95/2/EC on Food Additives other than Colours and Sweeterners. Office for Official Publications of the European Communities.

Paéz, B. C; Medina, A. R., Rubio, F. C., Moreno, P. G., Grima, E. M. **(2003).** Modeling the effect of free water on enzyme activity in immobilized lipase-catalyzed reactions in organic solvents. *Enzyme Microb. Technol.* 33, 845-853.

Peng, L.; Xu, X.; Tan, T. **(2000).** Lipase-catalyzed production of high quantity monoglycerides. In *Research Advances in Oil Chemistry.* Ed. Mohan, R. M. GRN, Kerala, India, vol. 1, 53-78.

Peyrou, G.; Rakotondrazafy, V.; Moulonoungui, Z. **(1996).** Separation and quantitation of mono-di- and triglycerides and free oleic acid using thin-layer chromatography with flame-ionization detection. *Lipids*. 31, 27-32.

Plou, F. J.; Barandiarán, M.; Calvo, M. V.; Ballesteros, A.; Pastor, E. **(1996).** High-yield production of mono-and dioleyglycerol by lipase-catalyzed hydrolysis of triolein. *Enzyme Microb. Technol.* 18, 66-71.

Premlal Ranjith, H. M.; Wijewardene, U. **(2006).** Lipid emulsifiers and surfactants in dairy and bakery products. In *Modifying lipids for use in food.* Ed. Gunstone, F. D. Woodhead Publishing, England, 393-428.

Reddy, M. S.; Rao, C. V. **(1965).** Vapour-liquid equilibria: benzene-*tert*-butanol and cyclohexane-*tert*-butanol systems. *Indian J. Technol.* 3, 45-48.

Reetz; M. T. **(2002).** Lipases as practical biocatalysts. *Chem. Biol.* 6, 145-150.

Rendon, X.; Lopez-Munguia, A.; Castillo, E. **(2001).** Solvent engineering applied to lipase-catalyzed glycerolysis of triolein. *J. Am. Oil Chem. Soc.* 78 (10), 1061-1066.

Rosas-Romero, A. J.; Herrera, J. C., Muccini, M. **(1996).** Determination of neutral and polar lipids by thin layer chromatography with flame ionisation detection. *Ital. J. Food Sci.*, 33-39.

Rosu, R.; Uozaki, Y.; Iwasaki, Y.; Yamane, T. **(1997).** Repeated use of immobilized lipase for monoacylglycerol production by solid-phase glycerolysis of olive oil. *J. Am. Oil Chem. Soc.* 74 (4), 445-450.

Ruzzu, A.; Mathiss, B. **(2002).** Swelling of PMMA-structures in aqueous solutions and room temperature Ni-electroforming. *Microsyst.Technol.* 8, 116-119.

Shanta, N. C.; Napolitano G. E. **(1998).** Lipid analysis using thin-layer chromatography and the Iatroscan. In *Lipid Analysis in Oils and Fats.* Ed. Hamilton, R. J. Blackie Academic and Professional, Great Britain, 1-33.

Shimada, Y.; Watanabe, Y.; Nagao, H.; Sugihara, A.; Baba, T.; Oguri, T.; Moriyama, S. **(2004).** Patent JP2004168985: Omega-3 type highly unsaturated fatty acid-containing partial glyceride composition and its production.

Sioen, I.; Huybrechts, I.; Verbeke, W.; Camp, J van; Henauw, S. de **(2007).** n-6 and n-3 PUFA intakes of pre-school children in Flanders, Belgium. *Br. J. Nutr.* 98 (4), 819-825.

Stevenson, D. E.; Stanley, R. A.; Furneaux, R. H. **(1993).** Glycerolysis of Tallow with Immobilized Lipase. *Biotechnol. Lett.* 15 (10), 1043-1048.

Stoye, D. **(2008).** Solvents In *Ullmann's Encyclopedia of Industrial Chemistry.* Wiley-VCH, Weinheim, Germany. 7th ed.

Striby, L.; Lafont, R.; Goutx, M. **(1999).** Improvement in the Iatroscan thin-layer chromatographic-flame ionisation detection analysis of marine lipids. Separation and quantitation of monoacylglycerols and diacylglycerols in standards and natural samples. *J. Chromatogr. A.* 849 (2), 371-380.

Sugiura, M.; Yamaguchi, H.; Yamada, N. **(2001).** Patent EP1111064A1: Process for the enzymatic preparation of diglycerides.

Tan, T.; Yin, C. **(2005).** The mechanism and kinetic model for glycerolysis by 1,3-position specific lipase from *Rhizopus arrhizus. Biochem. Eng. J.* 25, 39-45.

Thude, S.; Shukun, L.; Said, M. B.; Bornscheuer, U. T. **(1997).** Lipase-catalyzed synthesis of monoacylglycerides by glycerolysis of campher tree seed oil and cocoa-butter. *Fett-Lipid.* 99 (7), 246-250.

Tominaga, Y.; Sugihara, A.; Shimada, Y.; Maruyama, K.; Shiina, C.; Nakayama, H. **(1996).** Patent JP8214892: Production of partial glyceride containing highly unsaturated fatty acid.

Tüter, M.; Arat, F.; Dandik, L.; Aksoy, H. A. **(1998).** Solvent-free glycerolysis of sunflower oil and anchovy oil catalyzed by a 1,3-specific lipase. *Biotechnol. Lett.* 20 (3), 291-294.

Tüter, M.; Babali, B.; Kose, O.; Dural, S.; Aksoy, H. A. **(1999).** Solvent-free glycerolysis of palm and palm kernel oils catalyzed by a 1,3-specific lipase and fatty acid composition of glycerolysis products. *Biotechnol. Lett.* 21 (3), 245-248.

Tüter, M.; Aksoy, H. A. **(2000).** Solvent-free glycerolysis of palm and palm kernel oils catalyzed by commercial 1,3-specific lipase from Humicola lanuginosa and composition of glycerolysis products. *Biotechnol. Lett.* 22 (1), 31-34.

Uppenberg, J.; Hansen, M. T.; Patkar, S.; Jones, T. A. **(1994).** The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida antartica. Structure* 2 (4), 293-308.

Vikbjerg, A. F.; Jonsson, G.; Mu, H.; Xu, X. **(2006)**. Application of ultrafiltration membranes for purification of structured phospholipids produced by lipase-catalyzed acidolysis. *Sep. Pur. Technol.* 50 (2), 184-191.

Watanabe, T.; Sugiura, M.; Sato, M.; Yamada, N.; Nakanishi, K. **(2005).** Diacylglycerol production in a packed bed bioreactor. *Process Biochem.*, 40, 637-643.

Weete, J. D. **(1998).** Microbial Lipases. In *Food Lipids.* Eds. Akoh, C. C.; Min, D. B. Marcel Dekker USA, 641-664.

Xu, X.; Balchen, S., Hoy, C. E.; Adler-Nissen, J. **(1998).** Production of specific-structured lipids by enzymatic interesterification in a pilot continuos enzyme bed reactor. *J. Am. Oil. Chem. Soc.* 75, 1573-1579.

Xu, X. **(2000).** Modification of Oils and Fats by Lipase-Catalyzed Interesterfication: Aspects of Process engineering. In *Enzymes in Lipid Modification.* Ed. Bornscheuer, U. T. Wiley-vch, Germany 190-215.

Xu, X. B.; Skands, A.; Jonsson, G.; Adler-Nissen, J. **(2000).** Production of structured lipids by lipase-catalysed interesterification in an ultrafiltration membrane reactor. *Biotechnol. Lett.* 22 (21), 1667-1671.

Xu, X. B.; Jacobsen, C.; Nielsen, N. S.; Heinrich, M. T.; Zhou, D. Q. **(2002a).** Purification and deodorization of structured lipids by short path distillation. *Eur. J. Lipid Sci. Technol.* 104 (11), 745-755.

Xu, X.; Porsgaard, T.; Zhang, H.; Adler-Nissen, J.; Høy, C.-E. **(2002b).** Production of Structured Lipids in a Packed-Bed Reactor with *Thermomyces lanuginosa* Lipase. *J. Am. Oil. Chem. Soc.* 79 (6), 561-565.

Xu, X. **(2002c).** Response surface methodology for experimental optimization. *Lipid Technol.* January, 17-19.

Xu, X. **(2003).** Engineering of Enzymatic Reactions and Reactors for Lipid Modification and Synthesis. *Eur. J. Lipid Sci. Tech.* 105, 289-304.

Xu, X. **(2005).** SPD for Lipid Processing. In *Healthful Lipids*, eds. Akoh, C. C.; Lai, O-M. AOCS Press, Champaign, Illinois, USA, 127-144.

Yamauchi, Y.; Yamamoto, T.; Tsutsumi, K.; Shimada, O.-S.; Watanabe, N.-S.; Sugihara, I.-S.; Tominaga, O.-S. **(2003).** Patent US2003/0130533A1: Conjugated fatty acids containing monoglycerides and process for producing them.

Yang, B. K.; Parkin, K. L. **(1994).** Monoacylglycerol Production from Butteroil by Glycerolysis with A Gel-Entrapped Microbial Lipase in Microaqueous Media. *J. Food Sci.* 59 (1), 47-52.

Yang, T.; Xu, X **(2003).** Final confidential report (TR 6434): Enzymatic Production of Monoacyl Glycerols.

Yang, T.; Rebsdorf, M.; Engelrud, U.; Xu, X. **(2005a).** Enzymatic Production of Monoacylglycerols Containing Polyunsaturated Fatty Acids Through an Efficient Glycerolysis System, *J. Agric. Food. Chem.* 53, 1475-1481.

Yang, T.; Rebsdorf, M.; Engelrud, U.; Xu, X. **(2005b).** Monoacylglycerol Synthesis via Enzymatic Glycerolysis using a Simple and Efficient Reaction System. *J. Food Lipids* 12 (4), 299–312.

Zaks, A.; Klibanov, A. M. **(1984).** Enzymatic Catalysis in Organic Media at 100°C. *Science* 224 (3224), 1249-1251.

Zaks, A.; Klibanov, A. M. **(1985).** Enzyme –catalyzed processes in organic solvents. *P. Natl. Acad. Sci.* 3192-3196.

Zaks, A.; Gross, A. **(1990).** Patent WO 90/13656: Enzymatic production of glycerides containing omega-3 fatty acids.

Zaks A. **(1991).** Enzymes in organic Solvents. In *Biocatalysis for Industry.* Ed. Dordick, J.S. Plenum Press, New York, USA, 161-180.

Zaks, A.; Gross, A. **(1994).** Patent US5316927: Production of monoglycerides by enzymatic transesterification.

Zhang, K.; Shi, G.; Xia, Y. **(2000).** Patent CN1244587: Technological process of utilizing lipase in solid phase catalytic glycerination of oil and grease.

Zhang, H. **(2004).** Process development for lipase-catalyzed interesterification. In *Lipase-catalyzed interesterification for margarine fat production.* Ph.D thesis, BioCentrum-DTU, Technical University of Denmark, 37-57.

PAPERS AND APPENDICES

Paper I

Damstrup, M.L., Jensen, T., Sparsø, F.V., Kiil, S.Z., Jensen, A.D., and Xu, X.

Solvent Optimization for Efficient Enzymatic Monoacylglycerol Production Based on a Glycerolysis Reaction.

J. Am. Oil. Chem. Soc. 2005, 82 (8): 559-564.

Solvent Optimization for Efficient Enzymatic Monoacylglycerol Production Based on a Glycerolysis Reaction

M.L. Damstrup[a], T. Jensen[a], F.V. Sparsø[c], S.Z. Kiil[b], A.D. Jensen[b], and X. Xu[a,*]

[a]Food Biotechnology and Engineering, BioCentrum-DTU, DK-2800 Kgs. Lyngby, Denmark, [b]Department of Chemical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark, and [c]DANISCO A/S, DK-8220 Brabrand, Denmark

ABSTRACT: This study was aimed at screening solvent systems of varying polarities to identify suitable solvents for efficient and practical enzymatic glycerolysis. Several pure solvents and solvent mixtures were screened in a batch reaction system consisting of glycerol, sunflower oil, and Novozym® 435 lipase. Out of 13 solvents tested, *tert*-butanol and *tert*-pentanol were the only pure solvents suitable for a fast glycerolysis reaction with an acceptably high formation of MAG. In these systems, MAG contents of 68–82% were achieved within a few hours. Mixtures of *tert*-butanol/hexane, *tert*-pentanol/hexane, and *tert*-butanol/*tert*-pentanol in varying ratios also gave high MAG contents (58–78%). The tertiary alcohols *tert*-butanol and *tert*-pentanol, or mixtures of one of them with hexane, seemed to be the best choice among the solvents tested with respect to reaction efficiency, practical industrial applications, and steric hydroxyl group hindrance, which suppresses the ester formation with FA.

Paper no. J11089 in *JAOCS 82*, 559–564 (August 2005).

KEY WORDS: *Candida antarctica* lipase, glycerolysis, monoacylglycerols, organic solvents, sunflower oil, and tertiary alcohols.

The worldwide production of emulsifiers is estimated at approximately 200,000–250,000 metric tons per year (1,2). MAG or mixtures with DAG account for approximately 75% of the emulsifier production and have various applications in different fields (2). In the food industry, MAG are widely used in bakery products, margarines, dairy products, and confectionary because of their emulsifying, stabilizing, and conditioning properties. They are also important in cosmetic and pharmaceutical industries as drug carriers and for consistency improvements in creams and lotions. Owing to their lubricating and plasticizing properties, MAG are also used in textile and fiber processing, and in the production of plastics (3–7).

Commercial MAG are widely manufactured by the glycerolysis of fats or oils. The glycerolysis reaction is accelerated by the use of inorganic alkaline catalysts, such as NaOH or $Ca(OH)_2$, at high temperatures (220–260°C). The MAG content in the equilibrium mixture varies from 10 to 60%, depending on the glycerol-to-oil ratio in the reaction blend (2). Commercial synthesized glycerolysis processing usually provides a distribution between MAG, DAG, and TAG of 45–55, 38–45, and 8–12%, respectively (2). MAG are subsequently purified, typically by means of short-path distillation, to achieve a product of at least 90% purity (2,8–10).

This conventional process presents some disadvantages. The use of high temperature leads to the development of off-flavors and a dark color. It may also accelerate unwanted acyl migrations, in which FA residues migrate from one position of the glycerol backbone to another position [typically from the *sn*-2 position to the *sn*-1(3) positions] (2,4–8). Furthermore, the high-temperature chemical process is not suitable for the production of heat-sensitive MAG containing PUFA. Production of these heat-sensitive MAG is, however, of great commercial interest owing to their nutritional value, which could be applied in functional foods, pharmaceuticals, and the like.

Enzyme-catalyzed glycerolysis reactions are believed to be a potential alternative to the chemical process because of the much lower temperature required (below 80°C). The low temperature improves product quality and makes production of "new" heat-sensitive MAG feasible. Several investigations of low-temperature lipase-catalyzed glycerolysis have confirmed the potential of the enzyme-catalyzed processes, even though an industrial-scale process is still unavailable owing to the lack of efficiency (4–7,11–14). Long reaction times and/or low conversion of reactants, mostly attributable to the very poor miscibility of the hydrophilic glycerol and the lipophilic oil at low temperatures, make the reaction inefficient.

A suitable solvent system to improve the miscibility of substrates will result in a more homogeneous system and enhance the conversion of substrate, the reaction rate, and the product distribution in favor of MAG formation (7). Solvents such as *n*-hexane, *n*-heptane, dioxane, acetonitrile, acetone, isooctane, 2-methyl-2-propanol (*tert*-butanol), 2-methyl-2-butanol (*tert*-pentanol), or mixtures of some of them are useful in different lipase-catalyzed interesterification reactions. However, no careful solvent evaluation has been found in the literature concerning the enzymatic glycerolysis of vegetable oils (3,4,7,15–18).

This purpose of this study was to screen several pure and mixed solvents of varying polarities in order to evaluate solvents for an efficient enzymatic glycerolysis process with industrial applications. The reaction equilibrium should be achieved within a few hours; the reaction should favor the

*To whom correspondence should be addressed at BioCentrum-DTU, Technical University of Denmark, Bldg. 221, DK-2800 Kgs. Lyngby, Denmark. E-mail: mali@biocentrum.dtu.dk

Address reprint requests to Xuebing Xu, BioCentrum-DTU, Technical University of Denmark, Bldg. 221, DK-2800 Lyngby, Denmark.

formation of MAG; and the MAG content achieved should be at least as high as the chemical process. Solvents with different octanol–water partition coefficients (*P*), which are used in the literature as a parameter to characterize solvent properties in relation to lipids and lipases, were chosen (19). In general, *P* (or, in its more common form of expression, log *P*) provides a measure of the lipophilic vs. hydrophilic nature of a compound. *P* describes the distribution of a compound in a two-phase system and is defined as the ratio of the equilibrium concentration of the compound in a 1-octanol-rich phase to the concentration in a water-rich phase (in which water and 1-octanol are in equilibrium). Log *P* tends to be largest for compounds with extended nonpolar structures and smallest or negative for compounds with highly polar groups (20).

MATERIALS AND METHODS

Materials. Aarhus United (Aarhus, Denmark) provided the sunflower oil; this oil was a TAG oil with 97.1% TAG, 2.5% DAG, and 0.4% MAG and a water content of less than 0.01%. The FA composition (% w/w) of the sunflower oil was: C14:0, 0.1; C16:0, 6.7; C16:1, 0.2; C17:0, 0.1; C18:0, 3.7; C18:1, 26.3; C18:2, 61.2; C18:3, 0.4; C20:0, 0.3; C20:1, 0.2; C22:0, 0.6; C24:0, 0.2. Glycerol was purchased from VWR International Ltd. (Albertslund, Denmark) (purity, 99.5% w/w). Novozymes A/S (Bagsvaerd, Denmark) supplied the lipase enzyme Novozym® 435. Novozym 435, which originates from *Candida antarctica* and is produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism, is subsequently adsorbed on a macroporous resin. All solvents used were of analytical grade with a purity of 98–99.9% and were provided by Sigma-Aldrich (Broendby, Denmark).

Enzymatic glycerolysis reaction. Enzymatic glycerolysis was performed in batch experiments. Reaction blends for solvent screening consisted of 10.0 g sunflower oil, 5.26 g glycerol, and 50 mL solvent. Reaction blends for the time courses of the reaction consisted of 10.0 g sunflower oil, 4.21 g glycerol, and 40 mL solvent. The mixtures were incubated in capped 80-mL flasks in a water bath with magnetic stirring at 50°C. The reaction was initiated by addition of 3.0 g lipase enzyme. After 150 min or the set time for solvent screening and the time course of the reaction, 2 mL of the reaction mixture was withdrawn and filtered through a syringe filled with cotton wool to remove the enzyme. Subsequently, samples were flushed with nitrogen to remove the solvent. All samples were stored at −20°C prior to analysis.

GC analysis. The distribution of compounds in the mixture was analyzed by a PerkinElmer Autosystem 9000 capillary gas chromatograph equipped with a FID and a wall-coated open tubular fused-silica column (CP Sil 8 CB; 12.5 m × 0.25 mm i.d. × 0.1 μm film thickness of 5% phenyl-methyl-silicone; Chrompack, Middelburg, The Netherlands). The sample (50 mg) was dissolved in 12 mL heptane/pyridine (2:1, vol/vol) containing heptadecane (0.5 mg/mL) as an internal standard. The 500-μL sample solution was transferred to a crimp-capped vial; 100 μL of *N*,*N*-bis-trimethylsilyl-trifluoroacetamide was added and reacted for 15 min at 60°C. The oven temperature was programmed from 70 to 240°C at a rate of 15°C/min, increased to 350°C at a rate of 10°C/min, and held for 12 min. A 1.0 μL sample was then injected by programmed split-splitless injection cold split injection (initial temperature: 50°C; heated to 385°C) in a helium carrier gas and detected by FID at 395°C. The compounds were identified by comparing their retention times with those of authentic internal standards from DANISCO A/S (Brabrand, Denmark). Results were calculated based on response factors for MAG, DAG, TAG, and FFA from the standard and expressed as weight percentages.

TLC-FID analysis. Lipid profiles (MAG, DAG, and TAG) were analyzed by an Iatroscan MK6 TLC-FID system (SES GmbH, Bechenheim, Germany). Samples (20 mg) were diluted in 1 mL chloroform/methanol (85:15 vol/vol). Then 1 μL of diluted sample was spotted onto silica-coated Chromarod® quartz rods by a semiautomatic sample spotter, Model SES 3202/IS-02. Samples were developed for 25 min in a DT-150 development tank with *n*-heptane/diethyl ether/acetic acid 35:35:1 (by vol) as the solvent for development. The rods were dried for 5 min at 120°C in a TK-8 chromarod dryer before being measured on the Iatroscan MK6. Data handling was performed on a PC equipped with SES I-Chromstar® 6.0 software. Standard curves based on straight lines between weight and area (FID response) were constructed for external standards of monoolein (MAG), diolein (DAG), and triolein (TAG) from Larodan Fine Chemicals (Malmö, Sweden). MAG contents were calculated based on the standard curves and expressed as weight percentages of the sum of MAG + DAG + TAG. Content values are reported as the means of duplicate determinations.

Water content. Water content was analyzed by volumetric Karl Fischer titration by a Model 701 KF Metrohm titrator (Titrino, Bie & Berntsen, Roedovre, Denmark). Approximately 2 g of sample was added directly to a cell filled with methanol as solvent. A one-component reagent, Hydranal®-Composite 5, with a titer value of 5.2234 mg/mL, was used for titration. The reagent contained all the reactants (i.e., iodine, sulfur dioxide, and imidazole), which were dissolved in diethyleneglycol monoethyl ether. Results were expressed as weight percentages. Water content values are reported as the means of duplicate determinations.

Analysis of equilibrium conditions by the UNIFAC method. The UNIFAC method enables liquid–liquid mixtures to be evaluated by checking the consistency and properties of the structural units in the mixture (subgroups/UNIFAC groups) from which the molecules are formed. The UNIFAC model described by Hansen *et al.* (21) was chosen to describe the excess Gibbs energy of a liquid–liquid mixture of solvent in glycerol and triolein. ICAs version 7.0 software (Computer Aided Process Engineering Center, Kgs. Lyngby, Denmark) was used to assist in calculating the upper critical solution temperature (UCST) and the m.p. and b.p. UCST expresses the lowest temperature required to obtain a miscible mixture under equilibrium conditions and was calculated to verify the miscibility of selected solvents in both glycerol and oil (triolein) under set conditions (5–70°C). The m.p. and b.p. were calculated for se-

lected mixtures of solvents to estimate the displacement in properties compared with the pure compounds.

RESULTS AND DISCUSSION

Screening of pure solvents. Glycerolysis reactions were conducted in 13 different solvent systems with a range of log *P* values (Table 1). MAG formation in a solvent-free system was negligible after 150 min, as a result of the poor miscibility of glycerol and oil. Addition of a range of solvents such as alcohols and ketones undoubtedly enhanced the formation of MAG (Table 1). This is believed to be due to improvement in the miscibility of oil and glycerol, with resulting improvements in mass transfer. The tertiary alcohols improved MAG formation dramatically. With *tert*-butanol and *tert*-pentanol, it was possible to make a batch reaction system in which MAG contents as high as 83% (w/w) of lipids were achieved in a few hours.

Correlations between log *P* values and MAG contents after the glycerolysis reactions were done to investigate whether there was a pattern between the log *P* values of the solvents and the MAG formed (Fig. 1). The highest MAG contents were seen with solvents having log *P* values lower than one, and pronounced maximal yields were seen with log *P* values of 0.35 and 0.89 (*tert*-butanol and *tert*-pentanol, respectively) (Fig. 1). Hydrophilic solvents (such as isopropanol and ethanol, with log *P* values of 0.05 and –0.30) yielded higher MAG contents than hydrophobic solvents (such as *n*-heptane, *n*-hexane, and isooctane with log *P* values of 4.5, 4.0, and 5.15, respectively). However, the highest MAG content was obtained with *tert*-butanol and *tert*-pentanol as solvents. Reactions in 3-pentanone (log *P* value of 0.82) gave significantly lower MAG contents than in tertiary alcohols but higher contents than the most predominant hydrophilic and hydrophobic solvents. Thus, not only the polarity but also the functional groups played an important role in the solvent properties. The maximum MAG contents were obtained in solvents with log *P* values in the range of 0.3–1 and having a tertiary alcohol structure. The relatively low log *P* values of *tert*-butanol and *tert*-pentanol indicate both hydrophilic and hydrophobic characteristics, with predominantly hydrophilic characteristics. This is in accordance with the expectation that a solvent should have both water-like and octanol-like properties in order to be a suitable solvent for both oil and glycerol. Only *tert*-butanol and *tert*-pentanol were suitable solvents for an efficient industrial glycerolysis reaction with acceptably high contents of MAG.

The high content of MAG in the *tert*-butanol system agreed with Yang *et al.* (18). In that study, enzymatic production of MAG in *tert*-butanol was conducted at 40°C. They reached a MAG content of 60–70% (w/w) in the lipid phase in a stirred tank after a 2-h reaction. MAG contents up to 70% (w/w) of lipid phase [molar ratio of glycerol/oil (gly/oil) 4:1] were also reached in a packed bed reactor with a residence time of only 30–40 min (18).

Glycerolysis reactions in *tert*-butanol and *tert*-pentanol were further investigated by GC analysis. Typical time courses for MAG, DAG, and TAG in the two solvents systems are given in Figure 2. Equilibrium was reached after approximately 60 and 90 min in *tert*-pentanol and *tert*-butanol systems, respectively. The conversion of TAG was nearly complete at equilibrium conditions, and the equilibrium ratio of MAG/DAG was approximately 80:20 w/w%.

Alcohols can participate in lipase-catalyzed esterification reactions between FA and primary and secondary alcohols (23,24). Very little formation of unwanted FA esters took place (Table 2). The tertiary hydroxyl groups of the tertiary alcohols were sterically hindered toward the *C. antarctica* lipase and thus unable to form esters.

Unwanted FFA were formed during the reaction (Table 2). The formation of FFA indicates that water was present in the

TABLE 1
MAG Content After a Glycerolysis Reaction in Various Solvents and the Properties of the Solvents Screened

Solvent	MAG content[a]	Log *P* value[b]	m.p.[c] (°C)	b.p.[c] (°C)
Solvent free	0.0 ± 0.00			
Chloroform	0.0 ± 0.00	1.97	–63.41	61.17
n-Heptane	1.1 ± 0.02	4.50	–90.55	98.40
n-Hexane	1.4 ± 0.03	4.00	–95.35	68.73
Iso-octane	1.5 ± 0.17	5.15	–107.00	99.20
Acetonitrile	2.0 ± 0.07	–0.34	–43.82	81.65
Toluene	2.9 ± 0.20	2.73	–94.95	110.63
2-Butanone	5.4 ± 0.10	0.29	–86.64	79.59
Acetone	11.5 ± 0.73	–0.24	–94.70	56.05
Isopropanol	18.0 ± 0.31	0.05	–88.50	82.40
Ethanol	21.0 ± 0.18	–0.30	–114.14	78.29
3-Pentanone	29.4 ± 0.26	0.82	–39.00	101.00
tert-Pentanol	64.9 ± 1.12	0.89	–9.10	102.40
tert-Butanol	83.6 ± 0.14	0.35	25.69	82.40

[a]Weight percentages of lipids (MAG + DAG + TAG) ± SD. Reaction conditions: molar ratio glycerol/oil, 5:1; time, 150 min; temperature, 50°C; solvent, 50 mL/10 g oil; enzyme dosage, 30% (w/w) of oil.
[b]Based on data from Reference 20. Log *P* value, octanol–water partition coefficients.
[c]Based on data from Reference 22.

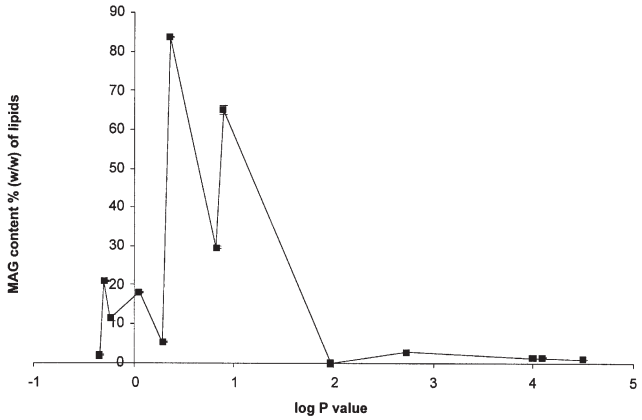


FIG. 1. Correlation between the log *P* value and MAG content achieved after the glycerolysis reaction. Reaction conditions: molar ratio glycerol/oil, 5:1; time, 150 min; temperature, 50°C; solvent, 50 mL/10 g oil; enzyme dosage, 30% (w/w) of oil. Error bars represent SD based on double determinations. Log *P* value, octanol–water partition coefficients.

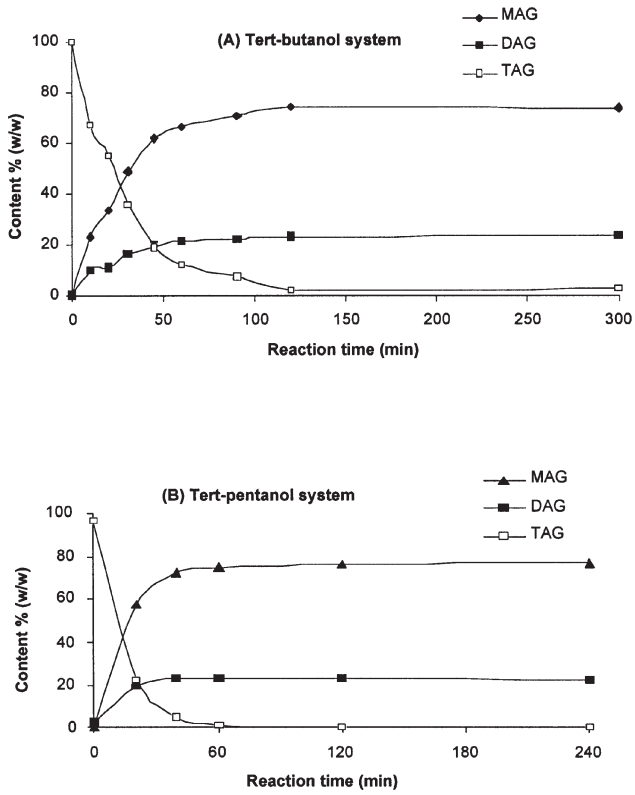


FIG. 2. Time course for the lipase-catalyzed glycerolysis reaction in two solvents: (A) *tert*-butanol, (B) *tert*-pentanol. Reaction conditions: Molar ratio glycerol/oil 4:1; temperature, 50°C; solvent, 40 mL/10 g oil; enzyme dosage, 30% (w/w) of oil.

reaction mixture. Since no water was added to the reaction mixture, the water originated from the reactants, i.e., solvent, enzyme, sunflower oil, and glycerol. The water contents of *tert*-butanol, *tert*-pentanol, Novozyme 435, sunflower oil, and glycerol were measured as 0.03, 0.09, 1.6, <0.01, and 0.06 wt%, respectively. These values were in accordance with the product descriptions from suppliers. Even these small amounts of water caused unwanted hydrolysis reactions. Identical substrates were used in both solvent systems, and the differences in water content found in the two solvents were negligible. Thus, no obvious explanation was found for the differences in FFA formation of 3.4 and 2.4% recorded for the *tert*-butanol and *tert*-pentanol systems.

In theory, a molar ratio (gly/oil) of 2 should result in optimal formation of MAG, but for several reasons this is not the case in enzyme-catalyzed glycerolysis. Higher molar ratios of gly/oil allow greater conversion of TAG to MAG. Therefore, in most reports of enzymatic glycerolysis, high ratios are used, and excessive quantities of unreacted glycerol are found at equilibrium (17). Reactions with a molar ratio (gly/oil) of 4 yielded approximately 60% MAG in an equilibrium mixture, corresponding to nearly 80% w/w MAG formation in the lipid phase (Table 2). This yield is undoubtedly very competitive with the yield of approximately 45–55 wt% for commercial chemical glycerolysis. The enzymatic glycerolysis process therefore seemed to be a very suitable alternative to the chemical process, not only in terms of the production of heat-sensitive compounds but also in terms of higher MAG contents. At equilibrium, 19% (w/w) unreacted/excess glycerol was obtained from the enzymatic glycerolysis with a molar ratio (gly/oil) of 4 (Table 2). Even with excess glycerol included in

TABLE 2
Composition of Reactant and Product Mixtures Before and After the Glycerolysis Reaction in *tert*-Pentanol and *tert*-Butanol Systems[a]

	tert-Pentanol system		*tert*-Butanol system	
Compound	Reactant mixture (wt%)	Product mixture (wt%)	Reactant mixture (wt%)	Product mixture (wt%)
FAE	0.1	1.9	0.0	0.6
FFA	0.3	2.7	0.8	4.2
MAG	0.3	59.1	0.3	57.3
DAG	1.9	17.2	1.9	17.8
TAG	74.0	0.4	73.7	0.5
Gly	23.4	18.7	23.3	19.6
Total	100	100	100	100

[a]Reaction conditions: molar ratio glycerol/oil 4:1; time, 240 min; temperature, 50°C; solvent, 50 mL/10 g oil; enzyme dosage, 30% (w/w) of oil. FAE, FA ester; Gly, glycerol.

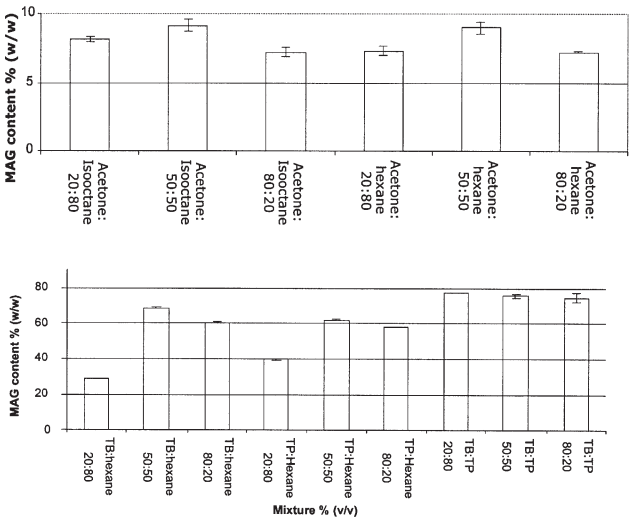


FIG. 3. MAG content [based on % (w/w) of MAG + DAG +TAG] for the lipase-catalyzed glycerolysis reaction in different solvent mixtures (% vol/vol). Reaction conditions: molar ratio glycerol/oil, 5:1; time, 150 min; temperature, 50°C; solvent, 50 mL/10 g oil; enzyme dosage, 30 % (w/w) of oil. Error bars represent SD based on double determinations. TB, *tert*-butanol/2-methyl-2-propanol; TP, *tert*-pentanol/2-methyl-2-butanol.

the calculations, the high MAG obtained makes the process very attractive.

The UCST of *tert*-butanol and *tert*-pentanol were calculated by the UNIFAC group contribution method (21) to verify the miscibility of *tert*-butanol and *tert*-pentanol with both glycerol and oil (triolein) in the temperature range at which the experiments were conducted. Surprisingly, glycerol and triolein were estimated to be immiscible in a *tert*-butanol system at temperatures below 78.1°C. Glycerol and triolein were estimated to be immiscible in a *tert*-pentanol system at temperatures below 28.1°C. The calculations indicated that a two-phase system occurred during the reaction with *tert*-butanol, although a two-phase system was not observed during the reaction, probably owing to strong agitation. However, samples withdrawn from the *tert*-butanol system separated into two phases after only a few minutes' storage. The phase separation was expected to lower the MAG formation because of mass transfer limitations, but it did not seem to be the case for this system.

Evaluation of solvent mixture for enzymatic glycerolysis. Two of the obvious advantages of using solvents are improved reactant homogeneity (one-phase system) and reduced reaction time. Further aspects of the solvents should be considered to find one suitable for industrial applications. Unwanted side reactions with substrates or products, as well as the cost, energy required for removal from the product mixture, toxicity, and the like are other important aspects to be considered. *tert*-Pentanol is much more expensive than *tert*-butanol, especially if a purity of 99% (w/w) is needed. Even though a technical-grade *tert*-pentanol is available at a reasonable price, the presence of 1–3% impurities, such as secondary alcohols, causes unwanted side reactions. Using short-path distillation to purify a reaction mixture containing *tert*-butanol can be difficult. The temperature range between the m.p. and b.p. of *tert*-butanol is very narrow, allowing a risk of solvent crystallization during condensation. When crystallization occurs, it can be difficult to condense the solvent, which worsens the possibility of reuse.

These factors have a negative impact on industrial suitability. The glycerolysis reaction was thus carried out in several solvent mixtures (Fig. 3). Yields of 58–80% occurred in mixtures of *tert*-butanol/hexane and *tert*-pentanol/hexane (up to 50% vol/vol hexane) and *tert*-butanol/*tert*-pentanol in varying ratios (20:80, 50:50, and 80:20% vol/vol). It was not possible to obtain an efficient glycerolysis reaction without addition of *tert*-butanol or *tert*-pentanol. However, it was possible to replace up to 50% of the tertiary alcohols with hexane. The results agree with a previous study (19), in which mixtures of *n*-hexane/*tert*-butanol was used successfully as solvents in lipase-catalyzed MAG synthesis. In that study, Monteiro *et al.* (19) conducted the esterification reaction and achieved a homogeneous one-phase system of lauric acid and glycerol in the presence of *n*-hexane/*tert*-butanol 1:1 (vol/vol).

TABLE 3
Properties and Price Indexes of Selected Solvent Mixtures

					Price index[c]	
Solvent mix[a]	Molar ratio	Ratio (vol/vol)	b.p.[b] (°C)	m.p.[b] (°C)	Supplier 1[d]	Supplier 2[e]
TB/TP	100:0	100:0	82.35	24.85	100	100
TB/TP	82:18	80:20	86.85	6.85	142	124
TB/TP	54:46	50:50	91.85	–30.15	204	160
TB/TP	23:77	20:80	95.85	–39.15	267	196
TB/TP	0:100	0:100	101.95	–9.15	309	220
Hexane/TB	100:0	100:0	68.75	–95.15	340	272
Hexane/TB	15:85	20:80	70.85	8.85	148	134
Hexane/TB	42:58	50:50	64.85	–11.15	220	186

[a]TB, *tert*-butanol; TP, *tert*-pentanol.
[b]Based on UNIFAC calculations. The m.p. was calculated for nonideal solutions.
[c]Calculated as the relative price index between identical solvent amounts and purities. A price index of 100 was set for the cheapest solvent combination.
[d]Data for price index calculations were based on solvent amounts of 5 L and purities of 99% (obtained from www.sigmaaldrich.com).
[e]Calculated as the relative price index between solvent amounts of 5 metric tons with a purity of 99% for TB and hexane and a purity of 98% for TP obtained from DANISCO A/S (Brabrand, Denmark). For abbreviations see Table 2.

The m.p. and b.p. for mixtures of *tert*-butanol/*tert*-pentanol and *tert*-butanol/hexane were calculated by the UNIFAC method to see the effect on properties of the solvent mixtures compared with the pure solvents. Furthermore, the price index was compared for the different mixtures (Table 3). It is possible to change the m.p. and b.p. to obtain temperature ranges that can be operated easily in terms of engineering during distillation. From an economic point of view, *tert*-butanol was found to be most beneficial compared with *tert*-pentanol and hexane, but a "reasonable" price was also found by substituting 20% (vol/vol) of *tert*-butanol with *n*-hexane or *tert*-pentanol.

Enzymatic glycerolysis in certain solvent systems has potential as an industrial process for the production of MAG. The tertiary alcohols *tert*-butanol and *tert*-pentanol, or one of them mixed with *n*-hexane, provided good reaction efficiency, practical industrial applications, and steric hindrance to undesired esterification reactions with the FA. With enzymatic glycerolysis in a solvent system, it was feasible to produce a high content of MAG at lower temperatures than those of the current chemical glycerolysis processes.

ACKNOWLEDGMENTS

Financial support from DANISCO A/S (Brabrand, Denmark) is gratefully acknowledged. Advice from Jens Abildskov and technical assistance from Kaare Joergensen, Department of Chemical Engineering-DTU, are appreciated.

REFERENCES

1. Moonen, H., and H. Bas, Mono- and Diglycerides, in *Emulsifiers in Food Technology,* edited by R.J. Whitehurst, Blackwell, Oxford, 2004, pp. 40–58.
2. Krog, N., Food Emulsifiers, in *Lipid Technologies and Applications,* edited by F.D. Gunstone, Marcel Dekker, New York, 1997, pp. 521–534.
3. Bellot, J.C., L. Choisnard, E. Castillo, and A. Marty, Combining Solvent Engineering and Thermodynamic Modeling to Enhance Selectivity During Monoglyceride Synthesis by Lipase-Catalyzed Esterification, *Enzyme Microb. Technol. 28*:362–369 (2001).
4. Elfman-Borjesson, I., and M. Harrod, Synthesis of Monoglycerides by Glycerolysis of Rapeseed Oil Using Immobilized Lipase, *J. Am. Oil Chem. Soc. 76*:701–707 (1999).
5. Ferreira-Dias, S., A.C. Correia, F.O. Baptista, and M.M.R. da Fonseca, Contribution of Response Surface Design to the Development of Glycerolysis Systems Catalyzed by Commercial Immobilized Lipases, *J. Mol. Catal. B–Enz. 11*:699–711 (2001).
6. Coteron, A., M. Martinez, and J. Aracil, Reactions of Olive Oil and Glycerol over Immobilized Lipases, *J. Am. Oil Chem. Soc 75*:657–660 (1998).
7. Kaewthong, W., and A.H. Kittikun, Glycerolysis of Palm Olein by Immobilized Lipase PS in Organic Solvents, *Enzyme Microb. Technol. 35*:218–222 (2004).
8. Bornscheuer, U.T., Lipase-Catalyzed Syntheses of Monoacylglycerols, *Ibid. 17*:578–586 (1995).
9. Xu, X., Modification of Oils and Fats by Lipase-Catalyzed Interesterification: Aspects of Process Engineering, in *Enzymes in Lipid Modification,* edited by U.T. Bornscheuer, Wiley-VCH, Weinheim, Germany, 2000, pp. 190–215.
10. Lee, G.-C., D.L. Wang, Y.-F. Ho, and J.-F. Shaw, Lipase-Catalyzed Alcoholysis of Triglycerides for Short-Chain Monoglyceride Production, *J. Am. Oil Chem. Soc 81*:533–536 (2004).
11. McNeill, G.P., and R.G. Berger, Enzymatic Glycerolysis of Palm Oil Fractions and a Palm Oil Based Model Mixture—Relationship Between Fatty-Acid Composition and Monoglyceride Yield, *Food Biotechnol. 7*:75–87 (1993).
12. Myrnes, B., H. Barstad, R.L. Olsen, and E.O. Elvevoll, Solvent-Free Enzymatic Glycerolysis of Marine Oils, *J. Am. Oil Chem. Soc. 72*:1339–1344 (1995).
13. Noureddini, H., and S.E. Harmeier, Enzymatic Glycerolysis of Soybean Oil, *Ibid. 75*:1359–1365 (1998).
14. Rosu, R., Y. Uozaki, Y. Iwasaki, and T. Yamane, Repeated Use of Immobilized Lipase for Monoacylglycerol Production by Solid-Phase Glycerolysis of Olive Oil, *Ibid. 74*:445–450 (1997).
15. Goto, M., N. Kamiya, and F. Nakashio, Enzymatic Interesterification of Triglyceride with Surfactant-Coated Lipase in Organic Media, *Biotechnol. Bioeng. 45*:27–32 (1995).
16. Hess, R., U. Bornscheuer, A. Capewell, and T. Scheper, Lipase-Catalyzed Synthesis of Monostearoylglycerol in Organic-Solvents from 1,2-*O*-Isopropylidene Glycerol, *Enzyme Microb. Technol 17*:725–728 (1995).
17. Rendon, X., A. Lopez-Munguia, and E. Castillo, Solvent Engineering Applied to Lipase-Catalyzed Glycerolysis of Triolein, *J. Am. Oil Chem. Soc. 78*:1061–1066 (2001).
18. Yang, T., M. Rebsdorf, U. Engelrud, and X. Xu, Enzymatic Production of Monoacylglycerols Containing Polyunsaturated Fatty Acids Through an Efficient Glycerolysis System, *J. Agric. Food. Chem. 53*:1475–1481 (2005).
19. Monteiro, J.B., M.G. Nascimento, and J.L. Ninow, Lipase-Catalyzed Synthesis of Monoacylglycerol in a Homogenous System, *Biotechnol. Lett. 25*:641–644 (2003).
20. Lide, D.R., Octanol–Water Partition Coefficients, in *Handbook of Chemistry and Physics,* 85th edn., edited by D.R. Lide, CRC Press, Boca Raton, FL, 2004–2005, section 16.
21. Hansen, H.K., P. Rasmussen, A. Fredenslund, M. Schiller, and J. Gmehling, Vapor–Liquid Equilibria by UNIFAC Group Contribution. V. Revision and Extension, *Ind. Eng. Chem. Res. 30*:2352–2355 (1991).
22. Lide, D.R., Physical Properties of Organic Compounds, in *Handbook of Chemistry and Physics*, 85th edn., edited by D.R. Lide, CRC Press, Boca Raton, FL, 2004—2005, section 3.
23. Gulati, R., P. Arya, B. Malhotra, A.K. Prasad, R.K. Saxena, J. Kumar, A.C. Watterson, and V.S. Parmar, Novel Biocatalytic Esterification Reactions on Fatty Acids: Synthesis of Sorbitol 1(6)-Monoesterate, *ARKIVOC 200*:159–170 (2003).
24. Xu, X., Engineering of Enzymatic Reactions and Reactors for Lipid Modification and Synthesis, *Eur. J. Lipid Sci. Technol. 105*:289–304 (2003).

[Received March 17, 2005; accepted June 20, 2005]

Paper II

Damstrup, M.L., Jensen, T., Sparsø, F.V., Kiil, S.Z., Jensen, A.D., and Xu, X.

Production of Heat-Sensitive Monoacylglycerols by Enzymatic Glycerolysis in *Tert*-Pentanol: Process Optimization by Response Surface Methodology.

J. Am. Oil. Chem. Soc. 2006, 83 (1): 27-33.

Production of Heat-Sensitive Monoacylglycerols by Enzymatic Glycerolysis in *tert*-Pentanol: Process Optimization by Response Surface Methodology

M.L. Damstrup[a,*], T. Jensen[a], F.V. Sparsø[c], S.Z. Kiil[b], A.D. Jensen[b], and X. Xu[a]

[a]Food Biotechnology and Engineering, BioCentrum-DTU, and [b]Department of Chemical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark; and [c]Danisco A/S, DK-8220 Brabrand, Denmark

ABSTRACT: The aim of this study was to optimize production of MAG by lipase-catalyzed glycerolysis in a tert-pentanol system. Twenty-nine batch reactions consisting of glycerol, sunflower oil, tert-pentanol, and commercially available lipase (Novozym®435) were carried out, with four process parameters being varied: Enzyme load, reaction time, substrate ratio of glycerol to oil, and solvent amount. Response surface methodology was applied to optimize the reaction system based on the experimental data achieved. MAG, DAG, and TAG contents, measured after a selected reaction time, were used as model responses. Well-fitting quadratic models were obtained for MAG, DAG, and TAG contents as a function of the process parameters with determination coefficients (R^2) of 0.89, 0.88, and 0.92, respectively. Of the main effects examined, only enzyme load and reaction time significantly influenced MAG, DAG, and TAG contents. Both enzyme amount and reaction time showed a surprisingly nonlinear relationship between factors (process parameters) and responses, indicating a local maximum. The substrate ratio of glycerol to oil did not significantly affect the MAG and TAG contents; however, it had a significant influence on DAG content. Contour plots were used to evaluate the optimal conditions for the complex interactions between the reaction parameters and responses. The optimal conditions established for MAG yield were: enzyme load, 18% (w/w of oil); glycerol/oil ratio, 7:1 (mol/mol); solvent amount, 500% (vol/wt of oil); and reaction time, 115 min. Under these conditions, a MAG content of 76% (w/w of lipid phase) was predicted. Verification experiments under optimized reaction conditions were conducted, and the results agreed well with the range of predictions.

Paper no. J11168 in *JAOCS 83*, 27–33 (January 2006).

KEY WORDS: *Candida antarctica* lipase, glycerolysis, monoacylglycerols, response surface methodology (RSM), sunflower oil, *tert*-pentanol.

MAG are amphiphilic molecules with both a hydrophilic and a hydrophobic part, giving excellent emulsifying properties. They are approved by the European Union (EU) as food-grade additives, have GRAS (Generally Recognized As Safe) status by the U.S. Food and Drug Administration, and can be used *quantis satis* (no maximum level permitted is specified) according to the European Parliament and Council Directive (1–3). Accordingly, MAG contribute to a large worldwide market and are found in varied applications, for example, food, cosmetics, pharmaceuticals, and plastic products (1,2).

Today, commercial MAG are manufactured by chemical glycerolysis of fats/oils and glycerol at high temperatures (220—250°C), using inorganic alkaline catalysts in a nitrogen gas atmosphere. The use of high temperature has some drawbacks, such as a dark color, burnt taste, and high energy consumption (1,2,4–7).

Commercial chemical glycerolysis usually provides 35–60% MAG, 35–50% DAG, 1-20% TAG, 1–10% FFA, and the alkali metal salts (1,2,4). According to the World Health Organization and the EU directive, MAG and DAG of FA are required to contain at least 70 wt% MAG + DAG, at least 30 wt% MAG, and a maximum of 7 wt% glycerol (8). To fulfill the requirements of the directives or achieve MAG products of even higher purity (90–95%), MAG are often purified from the equilibrium mixture by short-path distillation (9).

Lipase-catalyzed glycerolysis has attracted much interest in recent years (6,10–12). It is believed to be a potentially alternative method to chemical processing; one of the reasons being that a gentler technology with a much lower temperature is required (1,2,7–10,13). The low temperature below 80°C makes production of heat-sensitive MAG with PUFA feasible, which is difficult with the currently used chemical process. Thus, MAG from enzymatic glycerolysis offer industrial potential as ingredients or compounds with improved functionality or a healthier nutritional FA profile.

One of the main drawbacks of the low-temperature enzymatic glycerolysis reaction is that it comprises three phases: a hydrophobic oil phase, a hydrophilic glycerol phase, and a solid enzyme phase. Since enzymes have hydrophilic characteristics, glycerol often binds to the enzyme particles and makes access of the oil molecules to the enzyme difficult (11). As a result, MAG yield is relatively low, and the reaction time required may be impractical from an industrial point of view.

This has led to the use of bioconversions in "nonconventional" media to improve homogeneity and stability as well as to reduce viscosity and mass transfer limitations (1,7,11,14). Examinations of lipase-catalyzed interesterification reactions in different organic solvents such as dioxane, *n*-hexane, *n*-heptane, acetonitrile, acetone, isooctane, *tert*-butanol, and *tert*-pentanol confirm the benefits of nonconventional media (1,4,5,10,11,14).

*To whom correspondence should be addressed at BioCentrum-DTU, Technical University of Denmark, Bldg. 221, DK-2800 Lyngby, Denmark. E-mail: mali@biocentrum.dtu.dk.

Address reprint requests to xx@biocentrum.dtu.dk

Little effort has been made so far to optimize glycerolysis in *tert* pentanol. Addition of solvent lowers the space-time yield compared with a solvent-free system. However, tertiary alcohols, such as *tert*-pentanol, may enhance the reaction efficiency enough to offset the drawback of lowered productivity. High MAG yields after solvent and glycerol removal, as well as a well-preserved PUFA profile of the MAG, are among the benefits that can be obtained by enzymatic processing in solvent system (12). In addition, continuous operation and reusability of the solvent can overcome some of the problems with lowered productivity.

The present study is aimed at optimizing enzymatic glycerolysis in *tert*-pentanol to provide an industrially attractive reaction system for production of heat-sensitive MAG. Batch experiments, in which four process parameters are varied, are carried out and dataare evaluated by modeling with response surface design.

MATERIALS AND METHODS

Materials. Sunflower oil was provided by Aarhus United (Aarhus, Denmark). The sunflower oil was a TAG oil with 97.1% TAG, 2.5% DAG, 0.4% MAG, and a water content less than 0.01%. The FA composition of the sunflower oil was: C14:0, 0.1; C16:0, 6.7; C16:1, 0.2; C17:0, 0.1; C18:0, 3.7; C18:1, 26.3; C18:2, 61.2; C18:3, 0.4; C20:0, 0.3; C20:1, 0.2; C22:0, 0.6; C24:0, 0.2% (w/w). Glycerol was purchased from VWR International Ltd. (Albertslund, Denmark) (purity: 99.5 wt%). Novozymes A/S (Bagsværd, Denmark) supplied the lipase enzyme Novozym® 435. The enzyme is a *Candida antarctica* lipase that is produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism and that is subsequently adsorbed on a macroporous resin. *tert*-Pentanol was provided from Lab-Scan (Dublin, Ireland) (purity: 99%). Diethyl ether was provided from Bie & Berntsen A/S (Rødovre, Denmark) (purity: 99.5%). Chloroform (purity: 99.8%), methanol (purity: 99.8%), and *n*-heptane (purity: 95%) were provided from Lab-Scan. Acetic acid was purchased from VWR International Ltd. (purity: >90%).

Experimental design. The experimental work was designed according to the principle of response surface methodology (RSM) with the assistance of the commercial software, Modde 7.0 from Umetri (Umeå, Sweden). A three-level four-factor fractional experiment with five star points (29 experiments) was carried out. The four factors chosen were: enzyme load (w/w% of sunflower oil), reaction time (min), substrate ratio (glycerol/oil, mol/mol), and solvent amount (vol/wt% of oil). The measured MAG, DAG, and TAG contents were used as the responses. In Table 1 are listed the factors used, the parameter ranges applied, and the responses.

Enzymatic glycerolysis reaction. Enzymatic glycerolysis reactions in *tert*-pentanol were performed as batch experiments. Varied amounts of glycerol and *tert*-pentanol were mixed with 10 g of sunflower oil to obtain different substrate ratios (mol/mol) and solvent amounts (wt% of oil) in the system. Capped flasks containing the reaction mixtures were incubated in a water bath with magnetic stirring (Elektro, Helios) at 50°C. The reactions were initiated by addition of the lipase. After the set reaction time, 1.5 mL of reaction mixture was withdrawn and filtered through a syringe filled with water-repellent cotton to remove enzyme. Samples were flushed with nitrogen to remove air and solvent. All samples were stored at −20°C prior to analysis.

TLC-FID analysis. The lipid profiles (wt% of MAG, DAG, and TAG) were determined with an IATROSCAN MK6 TLC-FID (SES GmbH, Bechenheim, Germany). Silica gel-coated quartz rods (Chromarods-SIII, SESGmbH) were used (12). The dilution medium used was chloroform/methanol (85:15 vol/vol), and solvent was diethyl ether/heptane/acetic acid (35:35:1 by vol). The method is described elsewhere (12).

Theoretical product distribution. The theoretical product distributions of MAG, DAG, TAG, and glycerol at equilibrium conditions after glycerolysis reaction with different molar ratios of glycerol to oil were calculated in Microsoft Excel XP 2003/2002 and were based on binomial random distribution with probability calculations of ester formation between mole FA and mole hydroxyl groups (OH) calculated as $p_{ester} = {}^{n}FA/{}^{n}OH$. The mol% glycerol in the mixture with no esters had a b(0|3;p_{ester}) distribution. The mol% MAG in the mixture with one ester formed had a b(1|3;p_{ester}) distribution. The mol% DAG in the mixture with two esters formed had a b(2|3;p_{ester}) distribution. The mol% TAG in the mixture with two esters formed had a b(3|3;p_{ester}) distribution.

Statistical analysis. The experimental data were analyzed by means of RSM with Modde 7.0. Second-order coefficients were generated by regression with backward elimination. Responses were initially fitted to the factors by multiple regressions. The fit of the model was evaluated by the determination coefficients R^2 and Q^2 and the ANOVA. The insignificant coefficients were eliminated and the model was finally refined. The quadratic response surface model was fitted to the following equation:

$$Y = \hat{a}_0 + \sum_{i=1}^{4} \hat{a}_i x_i + \sum_{i=1}^{4} \hat{a}_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \hat{a}_{ij} x_i x_j \quad [1]$$

Y indicates the response variables (MAG, DAG, and TAG content, wt%), x_i (i = 1–4) the i^{th} independent variable, β_0 the intercept, β_i the first-order model coefficient, β_{ii} the quadratic coefficient for the i^{th} variable, and β_{ij} the linear model coefficient for the interaction between factor i and j (15).

RESULTS AND DISCUSSION

Preliminary evaluation of parameters. Preliminary studies were conducted to evaluate the ranges applied of selected parameters for RSM experiments. The MAG contents were evaluated during the time course of glycerolysis in the *tert*-pentanol system at different substrate ratios and solvent amounts (Fig. 1).

According to binomial distribution of MAG, DAG, TAG, and glycerol at equilibrium conditions after glycerolysis reaction with different molar ratios of glycerol to oil, increased MAG content was expected with increased molar ratio

TABLE 1
Factors for the Experiments Carried Out Based on a Four-Factor, Three-Level Surface Response Design and the Responses Achieved After Analysis on TLC-FID

	Factors[a]				Responses[b]		
Experiment no.	Enz.	Sol.	Sub.	Time	MAG	DAG	TAG
1	10	300	4	60	51.96	14.94	33.10
2	20	300	4	60	67.01	21.04	11.95
3	10	500	4	60	44.31	13.57	42.13
4	20	500	4	60	66.93	19.11	13.96
5	10	300	6	60	37.80	8.64	53.55
6	20	300	6	60	59.18	14.23	26.60
7	10	500	6	60	37.95	8.78	53.27
8	20	500	6	60	67.55	14.03	18.42
9	10	300	4	120	63.11	19.88	17.00
10	20	300	4	120	70.48	22.84	6.68
11	10	500	4	120	65.47	16.94	17.58
12	20	500	4	120	74.13	20.36	5.51
13	10	300	6	120	62.93	10.45	26.62
14	20	300	6	120	41.20	11.07	47.72
15	10	500	6	120	67.02	12.80	20.18
16	20	500	6	120	76.37	14.56	9.07
17	5	400	5	90	33.65	12.94	53.41
18	25	400	5	90	66.88	30.25	2.88
19	15	200	5	90	65.66	15.36	18.97
20	15	600	5	90	64.92	16.37	18.71
21	15	400	3	90	57.36	24.70	17.93
22	15	400	7	90	77.46	15.10	7.44
23	15	400	5	30	35.14	9.71	55.15
24	15	400	5	150	69.90	17.06	13.04
25	15	400	5	90	69.37	17.71	12.92
26	15	400	5	90	71.35	17.44	11.21
27	15	400	5	90	65.73	21.64	12.64
28	15	400	5	90	63.83	17.07	19.10
29	15	400	5	90	67.08	17.16	15.76

[a]Enz. = enzyme dosage (w/w of oil amount), Sol. = solvent amount (vol/wt of oil), Sub. = substrate ratio glycerol/oil (mol/mol), and Time in minutes.
[b]Responses of MAG, DAG, and TAG are calculated as weight percentages of the oil phase (MAG + DAG + TAG = 100%).

(Fig. 2A). Surprisingly, increased MAG formation was not seen with increased substrate ratio at ratios higher than six (all yielded MAG contents of approximately 80%) (Fig. 1). According to the binomial distribution calculations, an optimal MAG content, based on the complete product mixture including glycerol, was achieved at a substrate ratio (glycerol to oil) of 5 (mol/mol) (Fig. 2B). Hence, it was preferable to choose a substrate ratio close to 5. This explains why a glycerol to oil ratio of less than 7 (mol/mol) was suitable for further investigations. Equilibrium conditions were achieved after 120 min for all substrate ratios (Fig. 1). There was a tendency toward longer reaction time needed with increasing substrate ratios before equilibrium conditions were reached (Fig. 1). The intention was to set up a reaction system with shorter reaction time, but equilibrium should preferably be reached before the reaction was stopped. Thus, reaction times up to 150 min were selected to achieve maximal MAG formation.

Increasing the solvent amount seemed to enhance the formation of MAG (Fig. 1). This could be due to changes in the polarity of the system caused by the solvent. A greater amount of polar solvents might be a factor for increasing the catalytic activity because of the high polarity of the enzymes. Thus, variations in the amounts of solvents were of interest.

Calculated vs. experimentally achieved values. Experimental data with a reaction time of 90 min (Table 1) were compared with theoretical calculated equilibrium values (Fig. 2C). Experimental data agreed well with calculated values, showing that equilibrium indeed was reached after 90 min. Thus, only very short reaction times were required, confirming the greater efficiency of the *tert*-pentanol system compared with other enzymatic glycerolysis systems such as solvent free, solid-phase systems, where much longer reaction times typically are required (5,13).

Model fitting. Modeling of factors and responses was performed by RSM to predict the highest possible content of MAG. The underlying results for the models are listed in Table 1. A central composite rotatable design is generally the best design for response surface optimization (15). The best-fitting model was determined by regression and backward elimination. According to the models, the generated MAG, DAG, and TAG contents were affected by first-order variables (main effects) as well as second-order variables (interactions). All model coefficients (β) and probability values (P) were below

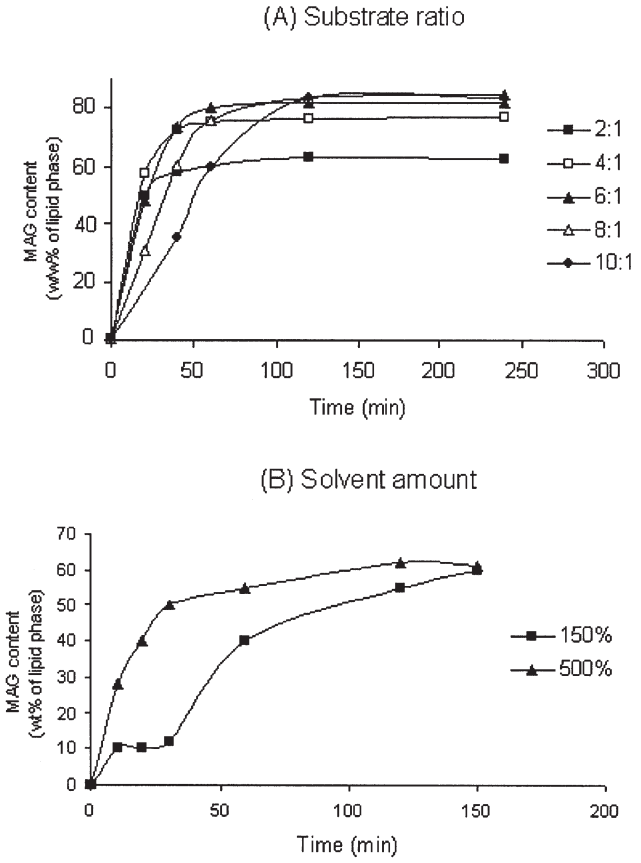


FIG. 1. Time course for MAG content during glycerolysis reaction in *tert*-pentanol system with: (A) varying substrate ratios of glycerol/oil (mol/mol), and (B) different solvent amounts. Reaction conditions: Enzyme dosage 30 wt% of oil, reaction temperature, 50°C. (A) Solvent amount, 200% (vol/wt of oil); (B) glycerol/oil ratio 5 (mol/mol).

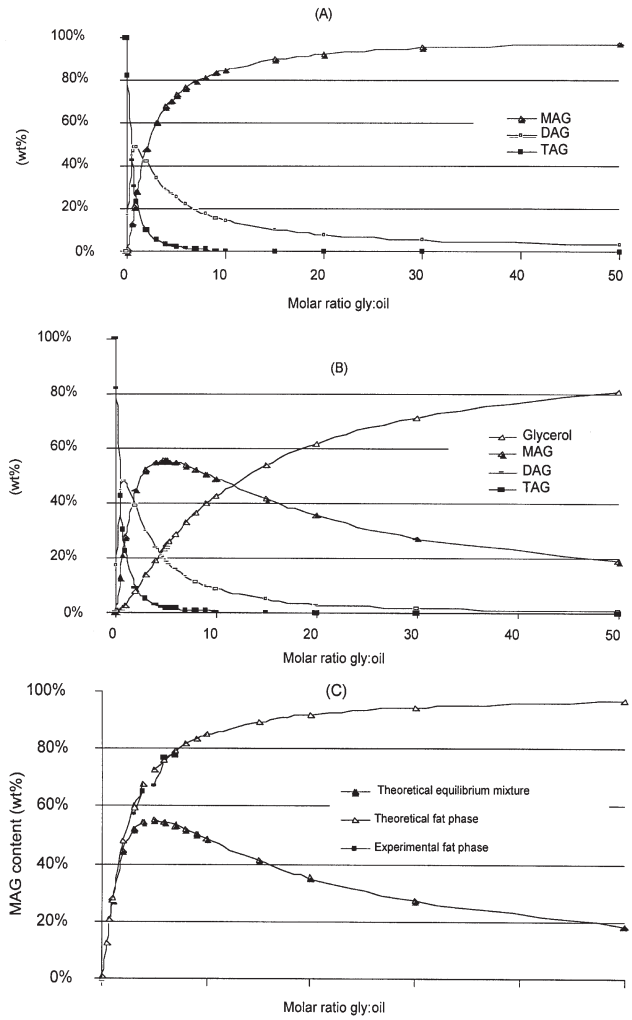


FIG. 2. Theoretical calculated product distribution at equilibrium conditions after glycerolysis reaction with different molar ratios of glycerol to oil. (A) Distribution between MAG, DAG, and TAG (fat phase), (B) distribution in the complete product mixture (fat phase and glycerol), and (C) theoretical and experimental MAG content in fat phase compared with the theoretical MAG content in complete product mixture including glycerol. Theoretical calculations are based on binomial random distribution with probability calculations of ester formation between mole FA and mole hydroxyl groups (OH), expressed as weight percentages.

0.05 after the models were refined (Table 2). ANOVA demonstrated that models were satisfactory with a coefficient of determination (R^2) for MAG, DAG, and TAG contents of 0.89, 0.88, and 0.92, respectively. The observed and predicted values were sufficiently correlated except for no. 14 (Fig. 3). No. 14 was treated as an outlier and eliminated; thereafter there was no lack of fit according to ANOVA. The successful fits indicate that models represent an actual relationship of reaction parameters within the ranges selected. It should be noted that the polynomials were only a statistical empirical model in the selected ranges and may not be true beyond the ranges of factors.

Main effects of parameters. The major influence of parameters can be evaluated from plots of main effects on MAG, DAG, and TAG contents. Enzyme amount and reaction time were the only two factors tested that significantly influenced MAG, DAG, and TAG content at the same time (Table 2). The first-order coefficients that had a positive effect on the MAG content had the opposite effect for the second-order coefficients. This indicates, surprisingly, a nonlinear relationship between factors and responses with optima. Increased enzyme load resulted in increased MAG content, until an optimal MAG content of 75% was obtained with an enzyme load of 20 wt% (Fig. 4). The MAG content was improved by longer reaction time until an optimum was obtained after 120 min with a MAG content of 76%. Instead of optima, equilibrium conditions with constant amounts of MAG, DAG, and TAG were expected after a certain reaction time and enzyme dosage in the selected enzyme range. The spreads in data at an enzyme dosage of 25% and reaction time of 150 min are relatively high and do not significantly differ from optimum enzyme dosage of 20% and reaction time of 120 min (Fig. 4). Thus, the decrease in MAG content with a reaction time longer than 120 min and an enzyme dosage higher than 20% are ascribed to spread in the data.

Substrate ratio had only a minor influence on glycerolysis (Table 2), in accordance with preliminary results (Fig. 1). The effect of substrate ratio was only significant on DAG content,

TABLE 2
Multiple Linear Regression Coefficients (β) and *P*-Values Describing the Influences of Different Parameters on the MAG, DAG, and TAG Content[a]

	MAG		DAG		TAG	
Term	Regression coefficient (β)	*P*-value[b]	Regression coefficient (β)	*P*-value[b]	Regression coefficient (β)	*P*-value[b]
Constant	68.04	4.09×10^{-19}	18.31	7.99×10^{-16}	13.65	1.25×10^{-6}
Enzyme	7.96	4.06×10^{-7}	3.03	9.44×10^{-7}	–10.99	7.09×10^{-9}
Solvent	0.52	0.63	–0.33	0.45	–0.19	0.87
Substrate	0.79	0.46	–2.77	3.27×10^{-6}	1.98	9.34×10^{-2}
Time	7.91	4.43×10^{-7}	1.51	2.25×10^{-3}	–9.41	7.91×10^{-8}
Enz*Enz	–3.93	6.9×10^{-4}	0.49	0.23	3.44	3.43×10^{-3}
Solvent*Solvent	–0.17	0.86	–0.94	2.70×10^{-2}	1.11	0.29
Time*Time	–3.37	2.59×10^{-3}	–1.56	8.05×10^{-4}	4.93	1.28×10^{-4}
Enz*Time	–3.30	2.10×10^{-2}	–0.43	0.43	3.73	1.47×10^{-2}

[a]Content values reported are based on weight percentages of MAG + DAG + TAG.
[b]*P*-values are defined as the smallest level of significance leading to rejection of the null hypothesis. The main effect of each factor (linear and quadratic) and the interaction effects are statistically significant when *P*-value is <0.05.

whereas the effect on MAG and TAG was insignificant (Table 2). Since the distribution of the three components MAG, DAG, and TAG are related to each other, the substrate ratio was expected to have a significant influence on MAG and TAG as well. However, no obvious explanation was found for the differences in significance level. Surprisingly, more glycerol did not produce more MAG, as expected from binomial distribu-

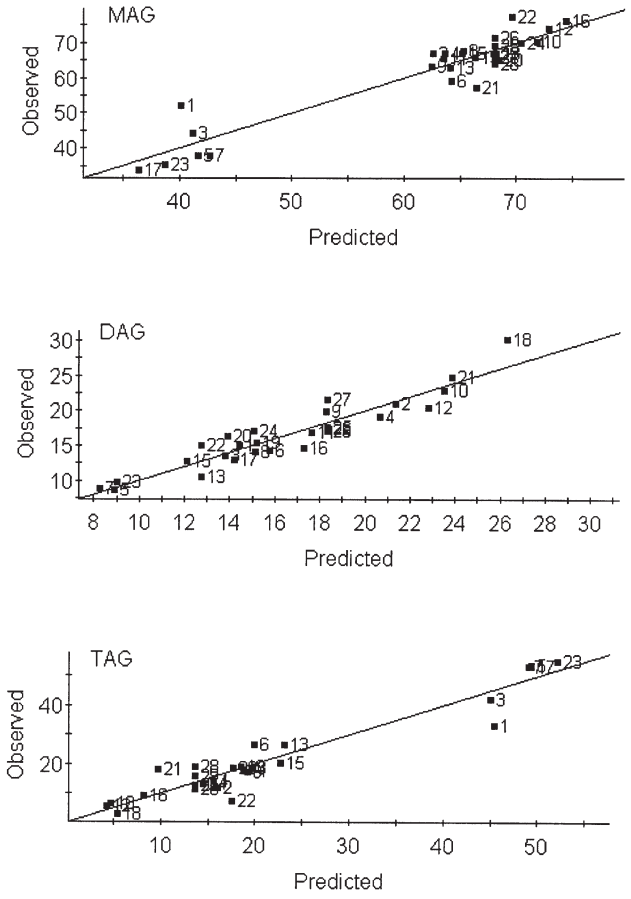


FIG. 3. Relationship between observed responses and results predicted by the developed model for MAG, DAG, and TAG content (w/w of lipid phase). Numbers inside the figures are experimental setting numbers. The solid line represents a linear regression line.

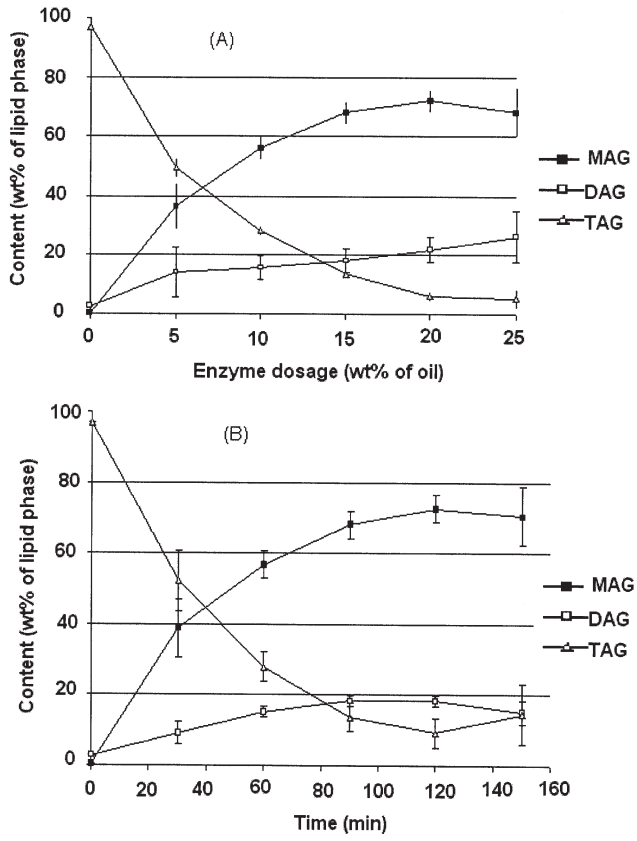


FIG. 4. Main effect of (A) enzyme dosage and (B) reaction time on MAG, DAG, and TAG formation at constant level of substrate ratio (glycerol to oil of 5 mol/mol), solvent amount (400 vol/wt of oil), and reaction temperature (50°C). Enzyme dosage in (B) was 15 (wt% of oil) and reaction time in (A) was 150 min. Error bars represent SD.

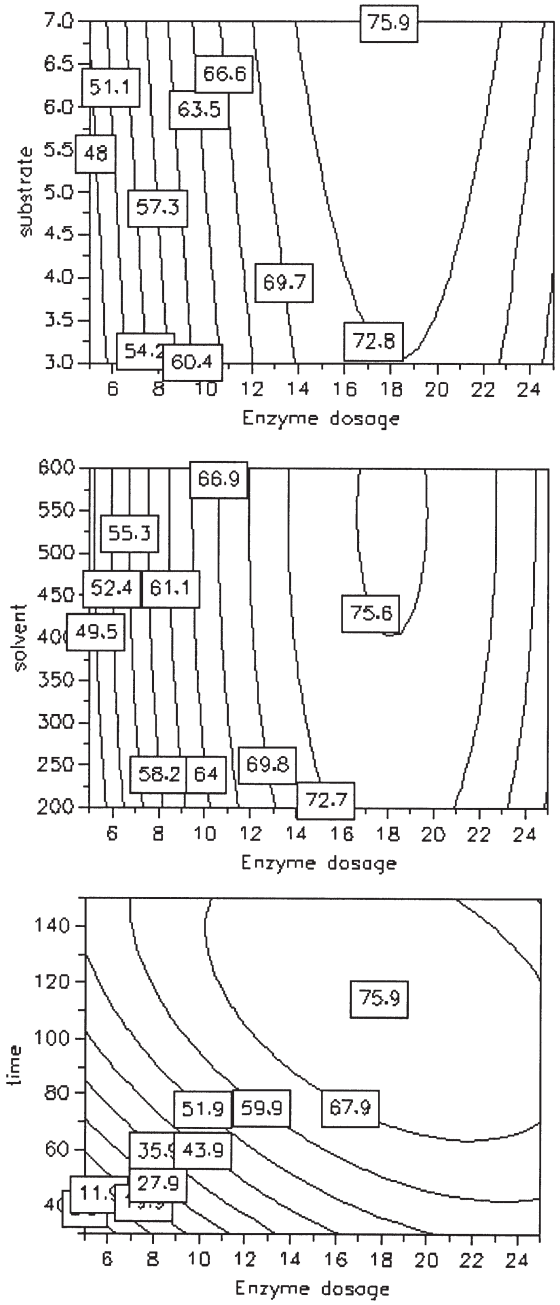


FIG. 5. Contour plots of MAG at optimized conditions. Enzyme dosage 18 wt%, solvent amount 500%, substrate ratio 7:1, and reaction time 115 min.

tion calculations (Fig. 2). No obvious explanation was found. The improved contact between glycerol and oil, caused by the solvent, might lead to higher efficiency, even at relatively low glycerol to oil ratios. Increasing the amount of glycerol changes the polarity of the system and renders plausible certain influences on the system stability and homogeneity. Apparently, an increased glycerol to oil ratio influenced the reaction in such a way that the expected stochiometric positive effects were neutralized.

The solvent amount was not crucial in batch reactions since the effect on MAG, DAG, and TAG contents was insignificant (Table 2). The effect of solvent amount was therefore neglected in the range tested (200–600% vol/wt). As a result, the system was not sensitive to reduced conversion with decreased solvent amount, either for reduced homogeneity or stability with reduced solvent amount. The conclusion with sufficient amount of solvent medium at both low and high solvent concentrations was not in complete agreement with preliminary results, in which the increasing amount of solvent affected the MAG content (Fig. 1). A slightly lower solvent amount of 150% was used in preliminary investigations. This indicates a lower critical solvent amount of approximately 200% before the polarity/homogeneity was affected negatively.

Optimization. According to the models generated, MAG, DAG, and TAG contents were influenced not only by first-order variables but also by second-order variables and parameter interactions. The complex relationship between reaction parameters and responses can be well evaluated by contour plots giving good predictions of optimized conditions. Several optimal combinations are available to obtain the highest MAG content. Contour plots between different parameters were generated for MAG formation. A pattern with high effect of enzyme dosage and reaction time and little effect of solvent amount and substrate ratio was seen (Fig. 5). The highest possible MAG content that could be established in this system was predicted to be 76 wt%, requiring an enzyme load of 18 wt%, substrate ratio of 7:1 (mol/mol), solvent amount of 500% (vol/wt of oil), and reaction time of 115 min. Verification experiments under optimized reaction conditions were conducted, and the results agreed well with the range of predictions.

Predicted optimal conditions must be interpreted in the context of industrial operations. The use of solvents increases expense, an extra process step for solvent removal is needed, and extra attention to safety issues is required as well. Accordingly, a solvent amount as low as possible is advantageous from an industrial point of view. A compromise in the solvent amount could easily be made without a dramatic reduction in the predicted MAG content (Fig. 5). A reduction of the solvent amount from 600 to 200% caused only a 3% reduction in the MAG content. A solvent amount lower than the predicted optimum is therefore recommended.

To fulfill the direct requirements of a maximum of 7 wt% glycerol in final products, the surplus of glycerol in the equilibrium mixture should be removed after the reaction. The predicted optimal glycerol to oil ratio of 7 theoretically provides excessive quantities of nonreacted glycerol in the equilibrium mixture of 42% (Fig. 2B). Removal of the excess glycerol is a main drawback from an industrial point of view because extra processing is required and it lowers the space-time yield of MAG in the product mixture. Since the influence of the substrate ratios on MAG content achieved was insignificant, a substrate ratio lower than the predicted optimum could easily be considered. A reduction of the substrate ratio from 7 to 3 caused only a 3% reduction in the MAG content (Fig. 5). Theoretically, this leads to a reduction in the nonreacted glycerol content from 42 to 24 wt% in the equilibrium mixture (Fig.

2B). However, at the same time, the DAG content increased from 15 to 25 wt% in the mixture (Table 1, Fig. 2B) and a high molar ratio of glycerol to oil allowed greater conversion of TAG to MAG and reduced the amount of DAG (Fig. 2). Although the increased DAG content did not affect the fulfillment of the directives of at least 70 wt% MAG + DAG and 30% MAG, it definitely had a negative impact on MAG purity and most likely on the product quality in general. Thus, the molar ratio of glycerol to oil should be very carefully selected depending on the required profile for the final product. To produce the highest possible MAG content in the lipid phase after solvent and glycerol removal, a high molar ratio of glycerol to oil was advantageous. On the other hand, to reach the highest MAG content in the complete product mixture (including glycerol, a molar ratio of approximately 5 was more appropriate. Industrial cost–benefits analysis must be performed to evaluate the optimized balance between product characteristics achieved, and the required processing.

The *tert*-pentanol system was successfully optimized through RSM. The empirical model developed satisfactorily expressed the MAG, DAG, and TAG formed during glycerolysis with regard to selected parameters. The high MAG content predicted showed an efficient glycerolysis system, even though some compromises in solvent amount and molar ratio were considered. The potential of *tert*-pentanol as a suitable solvent for low-temperature glycerolysis reaction was confirmed by the efficient optimized system that was developed.

ACKNOWLEDGMENT

Financial support from Danisco A/S, Brabrand, Denmark, is gratefully acknowledged.

REFERENCES

1. Yang, Y-C., S. Ramjan, and Y.-H. Ju, A Process for Synthesizing High Purity Monoglyceride, *J. Chin. Inst. Chem. Engrs. 34*:617–623 (2003).
2. Langone, M.A.P., M.E. De Abreu, M.J.C. Rezende, and G.L. Sant'Anna, Jr., Enzymatic Synthesis of Medium Chain Monoglycerides in a Solvent-free System, *Appl. Biochem. Biotechnol. 98*:987–996 (2002).
3. European Parliament and Council Directive No. 95/2/EC on Food Additives Other Than Colours and Sweeteners. http://europa.eu.int/eur-lex/en/consleg/pdf/1995/en_1995L0002_do_001.pdf (accessed April 2005).
4. Kwon, S.J., J.J. Han, and J.S. Rhee, Production and *in situ* Separation of Mono- or Diacylglycerol Catalyzed by Lipases in *n*-Hexane, *Enzyme Microb. Technol. 17*:700–704 (1995).
5. Bellot, J.C., L. Choisnard, E. Castillo, and A. Marty, Combining Solvent Engineering and Thermodynamic Modeling to Enhance Selectivity During Monoglyceride Synthesis by Lipase-Catalyzed Esterification, *Ibid. 28*:362–369 (2001).
6. Elfman-Borjesson, I., and M. Harrod, Synthesis of Monoglycerides by Glycerolysis of Rapeseed Oil Using Immobilized Lipase, *J. Am. Oil Chem. Soc. 76*:701–707 (1999).
7. Bornscheuer, U.T., Lipase-Catalyzed Syntheses of Monoacylglycerols, *Enzyme Microb. Technol. 17*:578–586 (1995).
8. European Food Emulsifier Manufactures Association, e.V. E 471, in *EFEMA Index of Food Emulsifiers*, 4th edn., 2004, pp. 51–55, http://www.emulsifiers.org (accessed April 2005).
9. Krog, N., Food Emulsifiers, in *Lipid Technologies and Applications,* edited by F.D. Gunstone, Marcel Dekker, New York, 1997, pp. 521–534.
10. Kaewthong, W., and A. H-Kittikun, Glycerolysis of Palm Olein by Immobilized Lipase PS in Organic Solvents, *Enzyme Microb. Technol. 35*:218–222 (2004).
11. Yang, T., M. Rebsdorf, U. Engelrud, and X. Xu, Enzymatic Production of Monoacylglycerols Containing Polyunsaturated Fatty Acids Through an Efficient Glycerolysis System, *J. Agric. Food Chem. 53*:1475–1481 (2005).
12. Damstrup, M.L., T. Jensen, F.V. Sparsø, S.Z. Kiil, A.D. Jensen, and X. Xu, Solvent Optimization for Efficient Enzymatic Monoacylglycerol Production Based on Glycerolysis Reaction, *J. Am. Oil Chem. Soc. 82*:559–564 (2005).
13. Chand, S., P. Adlercreutz, and B. Mattiasson, Lipase-Catalyzed Esterification of Ethylene Glycol to Mono- and Diesters. The Effect of Process Parameters on Reaction Rate and Product Distribution, *Enzyme Microb. Technol. 20*:102–106 (1997).
14. Roberts, N.J., A. Seago, J.S. Carey, R. Freer, C. Preston, and G.J. Lye, Lipase Catalysed Resolution of the Lotrafiban Intermediate 2,3,4,5-Tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic Acid Methyl Ester in Ionic Liquids: Comparison to the Industrial *t*-Butanol Process, *Green Chem. 6*:475–482 (2004).
15. Montgomery, D.C., Response Surface Methods and Other Approaches to Process Optimization, in *Design and Analysis of Experiments,* 4th edn., edited by D.C. Montgomery, John Wiley & Sons, New York, 1997, pp. 427–500.

[Received June 28, 2005; accepted October 20, 2005]

Paper III

Damstrup, M.L., Abildskov, J., Kiil, S., Jensen, A.D., Sparsø, F. V.; and Xu, X.

Evaluation of Binary Solvent Mixtures for Efficient Monoacylglycerol Production by Continuous Enzymatic Glycerolysis.

J. Agric. Food Chem. 2006, 54 (19): 7113-7119.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 7113−7119 **7113**

Evaluation of Binary Solvent Mixtures for Efficient Monoacylglycerol Production by Continuous Enzymatic Glycerolysis

MARIANNE L. DAMSTRUP,[†] JENS ABILDSKOV,[§] SØREN KIIL,[§] ANKER D. JENSEN,[§] FLEMMING V. SPARSØ,[#] AND XUEBING XU*,[†]

BioCentrum-DTU and Department of Chemical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark, and DANISCO A/S, DK-8220 Brabrand, Denmark

This study was aimed at evaluating different binary solvent mixtures for efficient industrial monoacylglycerol (MAG) production by enzymatic glycerolysis. Of all investigated cases, the binary mixture of *tert*-butanol:*tert*-pentanol (TB:TP) 80:20 vol % was the most suitable organic medium for continuous enzymatic glycerolysis, ensuring high MAG formation in a short time, reasonable solvent price, and easy handling during distillation/condensation processing. A minimum solvent dosage of 44−54 wt % of the reaction mixture was necessary to achieve high MAG yields of 47−56 wt %, within 20 min. The melting and boiling points of the TB:TP mixture were estimated to be 7 and 85 °C, respectively, using thermodynamic models. These predictions were in good agreement with experimentally determined values. In spite of the high reaction efficiency in the binary TB:TP system, the mixture of glycerol and sunflower oil (containing 97.1% triacylglycerol) yielded surprisingly a liquid/liquid phase split behavior even at high temperatures (>80 °C). This in contrast to thermodynamic model calculations suggested full miscibility in all proportions. These findings suggest that enhanced reaction efficiency in organic solvent also depends upon aspects other than the system homogeneity such as reduced viscosity, reduced mass transfer limitations, and the accessibility of the substrate to the active site of the enzyme.

KEYWORDS: ***Candida antarctica*** **lipase B; continuous reactor; glycerolysis; monoacylglycerols; tertiary alcohol media; vegetable oils**

INTRODUCTION

Monoacylglycerols (MAG) are extensively used emulsifiers in the food, pharmaceutical, and cosmetic industries (*1*, *2*). Besides bulk applications in the food and dairy industries, other applications of special MAGs with specific fatty acid profiles include medical, cosmetic, and hair care uses (*1*, *2*). The commercial interest for producing special MAGs containing, for instance, *n*-3 polyunsaturated fatty acid (PUFA) is increasing due to growing demands for healthier food lipid ingredients and other high-added-value products.

Currently, MAGs are widely manufactured by chemical glycerolysis of fats and oils at high temperature of 200−250 °C (*1*−*3*). Chemical glycerolysis suffers from the drawbacks of the high temperature, which leads to increase in flavor and color impurities (*1*−*3*). Handling of PUFA oil raw material with currently used methods is difficult due to the high rate of oxidization at high temperature (*4*). This has stimulated great interest in the gentler lipase-catalyzed glycerolysis process at ambient temperatures (*1*−*3*, *5*−*7*).

Vegetable oils are considered to be easily accessible, relatively cheap neutral triacylglycerol (TAG) raw material for carrying nutritionally important PUFAs. Thus, enzyme-catalyzed glycerolysis of vegetable PUFA oils seems to have strong industrial potential for improving MAG processing, quality, and functionality and thereby expanding MAG applications in functional foods and pharmaceuticals, etc. (*6*).

In the glycerolysis reaction, the use of organic solvents is beneficial for creating a homogeneous reaction system of the immiscible reactants glycerol and oil and to facilitate continuous reactor processes (*1*, *2*). Enzymatic glycerolysis in the organic solvents *tert*-butanol (TB) or *tert*-pentanol (TP) has previously been investigated and found to be very efficient for the production of MAG mixtures with PUFA profile (*5*−*7*). The two organic media enhance enzyme activity and accelerate the reaction rate dramatically compared to the solvent-free system (*5*−*7*).

However, from an industrial point of view, the use of binary solvent mixtures might be even more advantageous than that of pure solvents for glycerolysis. The use of pure TP (2-methyl-2-butanol/*tert*-amyl alcohol) has, for instance, the drawback of

* Address correspondence to this author at BioCentrum-DTU, Technical University of Denmark, Building 221, DK-2800 Lyngby, Denmark (telephone +45 4525 2636; e-mail mali@biocentrum.dtu.dk). Reprint requests should be directed to Xuebing Xu, BioCentrum-DTU, Technical University of Denmark, Building 221, DK-2800 Lyngby, Denmark (telephone +45 4525 2773; e-mail xx@biocentrum.dtu.dk).

[†] BioCentrum-DTU.

[§] Department of Chemical Engineering, Technical University of Denmark.

[#] DANISCO.

10.1021/jf061365r CCC: $33.50
Published on Web 08/29/2006

very high costs compared to pure TB (2-methyl-2-propanol/*tert*-butyl alcohol), especially at high purity of 99% (*6*). However, to avoid side reactions with solvent impurities, high solvent purity is necessary. Also, product purification is necessary to remove product impurities and achieve MAGs in highly pure form. MAGs in a pure form are advantageous to achieve emulsifying properties superior to those of MAG−diacylglycerol (DAG) mixtures (*1*, *2*). In the case of using pure TB, solvent removal and product purification by traditional distillation/condensation techniques is rendered difficult, because its melting and boiling points are quite close (57 °C) (*6*). Also, TB's melting point (25 °C) is quite high. This involves the risk of solvent crystallization during condensation of the evaporated solvent. Solvent crystallization in the equipment makes continuous operations difficult and limits the possibility for easy reuse of the solvent (*6*).

The present study aims to evaluate the behavior of industrial continuous enzymatic glycerolysis in mixed binary solvent systems and follows up on earlier investigations related to glycerolysis in organic media (*5*−*7*). Detailed investigations are carried out with emphasis on continuous operations, high MAG formation in a short time, low solvent price, a relatively broad melting−boiling profile of the solvent medium, and easy handling compared to selected pure solvent systems.

MATERIALS AND METHODS

Materials. Aarhus United, Aarhus, Denmark, provided sunflower oil, a triacylglycerol oil with a purity of 97.1%. The oil consisted mainly of unsaturated long-chain fatty acids: C16:0, 6.7 wt %; C18:1, 26.3 wt %; C18:2, 61.2 wt %. A more detailed description of the oil is found elsewhere (*5*, *6*). DANISCO A/S kindly provided DIMODANHR, a commercially available distilled MAG with a purity of ≥90%. Novozymes A/S, Bagsværd, Denmark, supplied Novozym435, a commercially available *Candida antarctica* lipase B (CALB) immobilized by physical adsorption onto a macroporous hydrophobic polymethyl methacrylate (PMMA) matrix. Glycerol with a purity of 99.5 wt % was purchased from VWR International Ltd., Albertslund, Denmark. All solvents used were of analytical grades with a purity of 96−99.7%. The solvents were purchased from Sigma-Aldrich, Brøndby, Denmark.

Thermodynamic Analysis. UNIFAC (*8*) was used to estimate the displacement in physical properties of selected binary solvent mixtures compared to the pure compounds. Melting and boiling points for the mixtures and pure components were calculated using isofugacity expressions for solid/liquid equilibrium and liquid/vapor equilibrium and are presented in phase diagrams. Further description of UNIFAC and UNIFAC calculations are given elsewhere (*8*).

Melting Point (MP) Measured by Differential Scanning Calorimetry (DSC). The transformation from solid to liquid of selected binary mixtures was studied by DSC (DSC 821e/HSS7, Mettler Toledo). Fifteen microliter samples were sealed in a ceramic sensor plate with a thermocouple that measured the difference between the heat flow to the sample and a reference. The samples were analyzed by initially cooling from 20 to −25 °C at 10 °C/min and held at −25 °C for 5 min. Subsequently, the samples were heated from −25 to 50 °C at a heating rate of 5 °C/min while onset, endset, offset and major peak maximum temperatures were measured. The melting point of the completely melted sample is determined as average values of triple determination of the endset values.

Continuous Glycerolysis in Packed Enzyme Column. A reaction mixture of sunflower oil, glycerol, and solvent was mixed in a feeding container/flask by magnetic stirring and preheated to 50 °C. The mixture, maintained at the set temperature and continuously stirred, was pumped (up-flow) through a column reactor (i.d. = 15 mm, o.d. = 38 mm, and length = 200 mm) with a Fluid Metering Inc. FMI Lab Pump QG150 (flow range = 0−16 mL/min) purchased from Micro Lab AARHUS A/S, Højbjerg, Denmark. The reactor consisted of a jacketed steel column dry packed with 8.0 g of enzyme, corresponding to 227 kg of enzyme/m^3 column. A weight-based reaction time was used defined as

$$\tau = \frac{W}{\nu_0 \rho} \quad (1)$$

where τ is the weight reaction time (min), W is the weight of catalyst pellets on a dry matter basis (g), ρ is the bulk density of the dry enzyme (g/cm^3), and ν_0 is the volumetric flow rate of reactant mixture (cm^3/min = flow rate/cross section of column). The column temperature was maintained at 40 °C by a water bath circulation. The column was initially stabilized, and bubbles were removed by running reaction mixture through the column equivalent to at least 3 times the reactor volume. The first product from the enzyme bed, equal to at least 4 times the weight time, was discarded to ensure the product outlet was representative. Samples were thereafter withdrawn and stored at −20 °C prior to analysis by gas chromatography (GC), which is described elsewhere (*6*). Results from GC analysis are expressed as weight percentages of the product mixture of glycerol, mono-, di-, and triacylglycerols, free fatty acids, and fatty acid esters.

Screening of Different Binary Mixture for Continuous Glycerolysis. Glycerol was mixed with sunflower oil, equal to a molar ratio of 5. Four different binary solvent mixtures were tested: TB:TP 80:20 and 90:10 vol %, TB:cyclohexane (cH) 80:20 vol % and TB:*n*-hexane (nH) 80:20 vol %, each mixture in an amount of 20 mL/10 g of oil. The purities of the solvents were as follows: TP, 96%; cH, 99%, nH, 97%; and TB, 99%. The flow rate was 0.6 mL/min, corresponding to laminar flow and a weight-based reaction time of 30 min. Results are expressed as average values of triple determination of samples withdrawn after 4, 7, and 24 h.

Effect of Solvent Dosage in Continuous Glycerolysis. Glycerol was mixed with sunflower oil, equal to a molar ratio of 3 and a binary mixture of TB:TP 80:20 vol % in four different dosages (28, 44, 54, and 61 wt % of reaction mixture). The purities of the TP and TB were 99 and 99.7%, respectively. The flow rates were 0.47 and 0.93 mL/min, corresponding to laminar flow and reaction−weight times of 20 and 40 min. Results are expressed as average values of double determination of samples withdrawn after 4 and 6 times the weight time.

Statistical Analysis. The Excel Analysis Toolpack: Analysis of Variances (ANOVA): Single Factor was used to test for significant differences between samples grouped by one variable (solvent combination or solvent dosage). The significance of the results was established at $P \leq 0.05$.

Phase Split Behavior of Reactant Mixtures. Reaction mixtures, with a total volume of 100 mL, were mixed of 50 wt % TB:TP 80:20 and 50 wt % glycerol plus oil in molar ratio of 3.7 or 5, respectively. The reaction mixtures were preheated to a selected temperature during magnetic stirring (40, 60, 75, 80, or 100 °C). When the selected temperature was reached, the magnetic stirrer was stopped, and the mixtures were left for 30 min and 24 h. Subsequently, the occurrence of a phase split was observed, indicating whether the reactants were miscible or not. The two different phases obtained from the reactant mixture of glycerol to oil molar ratio of 3.7 heated to 40 and 75 °C were withdrawn and collected in separate flasks. From each collected phase, 1 mL was used for GC analysis of the compound distribution, and the remaining part was used for analysis of the solvent distribution by rotation evaporation under vacuum at 70 °C. Results are based on double determinations.

Solvent Removal by Rotation Evaporation under Vacuum. A weighted sample amount (10−160 g) was heated in a water bath at a set temperature during rotation. The solvent was removed from the mixture by evaporation under vacuum at the selected temperature (60 or 73 °C). Subsequently, the distillate was condensed by cooling water and collected in a vessel (Büchi, Glasapparatefabrik Flawil, Switzerland). The solvent/byproduct collected was weighed, and the evaporated solvent was then calculated as weight percent of the initial solvent amount in the sample. Results are expressed as average values of double determinations.

RESULTS AND DISCUSSION

Predicted MP and BP of Different Binary Solvent Mixtures. MPs and BPs of binary TB:TP; TB:nH, and TB:cH mixtures can be evaluated through Txy (vapor/liquid equilibrium) and Tx (solid/liquid equilibrium) phase diagrams. Such phase diagrams were calculated using isofugacity relationships expressed in terms of UNIFAC activity coefficients as well as melting points, heats of melting, and vapor pressure relationships of the pure components. The resulting Tx diagrams are shown in **Figure 1a**, and the resulting Txy diagrams are shown in **Figure 1b.** For the solid/liquid systems, it is assumed that the two components are immiscible in the solid phase, so only pure solid phases are formed. This assumption leads to eutectic diagrams, with one branch corresponding to solidification of a single component at equilibrium with the (liquid) solvent mixture. The two branches coincide at the eutectic point/temperature. In general, two curves are shown per diagram: a lower curve resulting from assuming the liquid mixture to be an ideal solution and an upper resulting from taking into account liquid phase nonideality using UNIFAC activity coefficients and appropriate standard states. Perhaps not unexpectedly, the greatest differences between ideal mixture calculations and nonideal mixture calculations are found in the diagrams for alcohol/alkane systems. In the alcohol/alcohol (TB/TP) diagram the two curves are superimposed.

The binary solvent mixtures show in general broader/wider ranges between melting and boiling points than the pure TB system. The range between melting and boiling point of 57 °C for the pure TB was, for instance, enhanced to ranges of 78, 62, or 70 °C, when 20 vol% TP, normal hexane, or cyclohexane was added. Adding nH to TB lowers the melting and boiling points, with the MP depression effect dominating. Adding TP to TB lowers the MP and raises the BP. Adding cH to TB lowers the MP and does not alter the bubble point temperature significantly from the boiling temperature of TB.

Partial substitution of TB is desirable to avoid the risk of crystallization during purification processing after glycerolysis reaction. However, the substitution should be minimized to maintain the high reaction efficiency and low costs in the pure TB system (*6*, *7*). A MP below approximately 15 °C and a temperature range between the melting and boiling points of at least 65 °C was deemed to be sufficient to avoid practical problems with crystallization. Thus, replacement of 10−20 vol % of the TB with either TP, nH, cH seems appropriate to be to obtain sustainable practical operations.

Comparison of Predicted and Experimentally Determined MP of Binary Solvent Mixtures. To validate the UNIFAC predictions, MPs were determined experimentally using DSC. **Table 1** compares experimental and predicted values with literature values. Also, the price index for the different solvent combinations is given. Measured MPs for pure and binary solvent mixtures agreed well with predicted as well as literature values. The changed melting/boiling profile for TB:TP 90:10 vol % compared to that of pure TB was only minor, still allowing the risk of crystallization during downstream processing. Thus, with respect to practical operation at industrial facilities, only binary mixtures with no more than 80 vol % TB seem to be suitable.

Highly flammable and harmful risks are related to all four different solvents tested (*12*−*15*). *n*-Hexane has the advantage of being accepted as a food-grade extraction medium by the international Food and Nutrition Board, supported by the U.S. Food and Drug Administration (FDA) (*16*). However, the low BP of nH at 68.7 °C (*9*) has the disadvantage of pronounced flammable risk and easy release of harmful volatiles at industrial facilities maintained at typical temperatures of 20−25 °C. Cyclohexane has better acceptance in the industry than *n*-hexane, reflected by a doubling of the maximum exposure limit of cH compared to nH (*12*−*15*). Further details about the exposure limits are found under Safety. This is due to cyclohexane's higher boiling point at 80.7 °C (*9*), which leads to reduced vapor solvent at room temperature and, thus, improved health and safety environment at practical handling. Even though food legalization aspects slightly favor the use of nH for partial substitution of TB, it is difficult to recommend one solvent over the others on the basis of hazard considerations only.

Comparison of industrial obtained prices shows that TB:cH 80:20 vol % is the most economical choice, whereas TB:nH 80:20 vol % is the most costly mixture. The price difference of 44% between the cheapest and most expensive solvent combination is of considerable commercial importance. However, it was not found to be reliable to exclude any of the binary mixtures without further cost−benefit analysis of other aspects such as space−time requirements and product yield in the different solvent mixtures.

Glycerolysis Reaction in Binary Solvent Mixtures. Continuous glycerolysis reaction efficiency was tested in selected binary solvent mixtures and compared to the efficiency in the pure TP. Results are shown in **Figure 2**. In general, high MAG contents of 45−56 wt % were achieved in all of the tested binary solvents and were in a range close to an expected equilibrium MAG content of approximately 55 wt % at glycerol to oil molar ratios of 5 (*5*). Of the four different solvent mixtures tested, glycerolysis conducted in 80:20 vol % TB:TP resulted in the significantly highest MAG yield and was similar to the yield achieved in pure TP. Hexane (normal as well as cyclo) is much less polar than TP (*17*). It is very plausible that a reduced polarity in the two TB:hexane systems has a negative impact on the behavior of the reactant mixture, resulting in the lower MAG formation. This might be due to a coarser and more unstable emulsion of glycerol and oil in the less polar medium and, hence, to diminished substrate access to the active site of the enzyme.

The significant variations in MAG content between the 80:20 and 90:10 vol % ratio of the TB:TP system cannot be explained by polarity variations. TP is only slightly less polar than TB due to one more carbon atom placed in the alcohol chain (*17*). Anyhow, the TB:TP 80:20 vol % seems to be very promising for an efficient glycerolysis reaction with reaction efficiency similar to the efficiency in the pure TP. Accordingly, on the basis of the melting/boiling profile, costs, and reaction efficiency, the TB:TP 80:20 vol % solvent system is regarded as a competitive alternative to the pure TP and TB system. The TB:TP 80:20 vol % mixture has the advantage of improved physical properties compared to the pure TB system and reduced costs compared to pure TP system.

Effect of Solvent Dosage and Reaction Time in the Column Reactor. To evaluate the impact of the solvent dosage on the reaction efficiency, glycerolysis was conducted in a column reactor using different solvent mixture dosages at two different reaction times. The MAG content obtained at different solvent dosages is given in **Figure 3**. In general, an increase in the MAG content was observed with increased solvent dosage and prolonged reaction time. Hardly any MAGs were formed in the solvent-free system, whereas 9−59% MAGs was formed in the solvent system with 28−61 wt % solvent. The impact of the solvent dosage on the MAG content was much more crucial

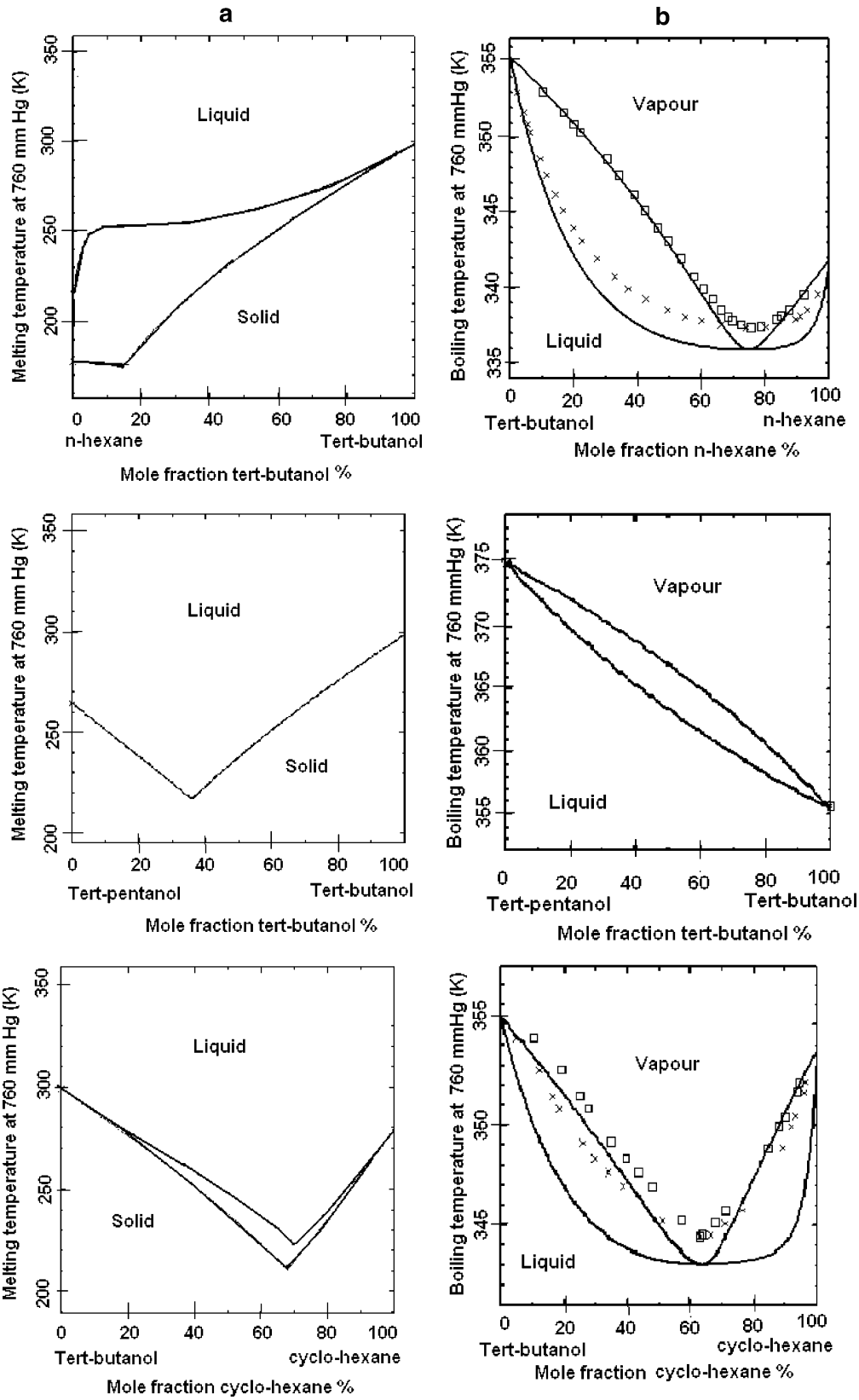


Figure 1. (**a**) Tx (solid/liquid) phase diagrams for binary mixtures of TB:*n*H, TP:TB, and TB:cH. The upper curve in the graphs represents a calculated nonideal solution behavior, and the lower curve represents a calculated ideal solution behavior. (**b**) Txy (vapor /liquid equilibrium) phase diagrams for binary mixtures of of TB:nH, TP:TB, and TB:cH. The curves represent calculated phase equilibrium for ideal solution behavior (lower curve) and nonideal solution behavior (upper curve). Discrete points represent known literature values.

in the lower solvent range from 0 to 44% compared to the upper range from 44 to 61%. At a solvent dosage of 28%, only a relatively "low" MAG content of 9−17% was achieved, whereas desirable "high" MAG contents of 46−59% were achieved at higher solvent dosages from 44 to 61 wt %. This indicates that a solvent dosage above a critical amount reacts as a kind of reaction activator, in this case between 28 and 44 wt % of the reaction mixture.

Table 1. Measured Melting Point for Different Pure and Binary Solvent Mixtures Compared to Predicted and Accessible Literature Values and Price Index for the Different Solvent Combinations Obtained from an Industrial Supplier

solvent 1:solvent 2	solvent ratio v/v	measured by DSC[a] MP (°C)	predicted by UNIFAC method (*8*) BP (°C)	predicted by UNIFAC method (*8*) MP (°C)	literature values BP (°C)	literature values MP (°C)	price index[b]
TB	100	21.9 ± 2.2	82	24.9	82.2 (*9*)	25.5 (*9*)	107
TP	100	−12.0 ± 0.6	101	−9.1	103 (*9*)	−9 (*9*)	235
(1) TB:TP	90–10	15.1 ± 1.4	83	17			120
(2) TB:TP	80–20	8.8 ± 0.9	85	7			132
(3) TB:nH	80–20	10.9 ± 0.6	72	10	73 (*10*)		144
(4) TB:cH	80–20	7.9 ± 0.5	77	7	75 (*11*)		100

[a] Average value of triple determinations. "Endset" values are used as expression for complete melted sample. [b] Calculated as relative price index between solvent amounts of 5 tons with purity of 99% for TB, *n*-hexane, and cyclohexane and purity of 98% for TP. Prices were obtained from DANISCO A/S, Brabrand, Denmark (2005). Price index = 100 is set for cheapest combination.

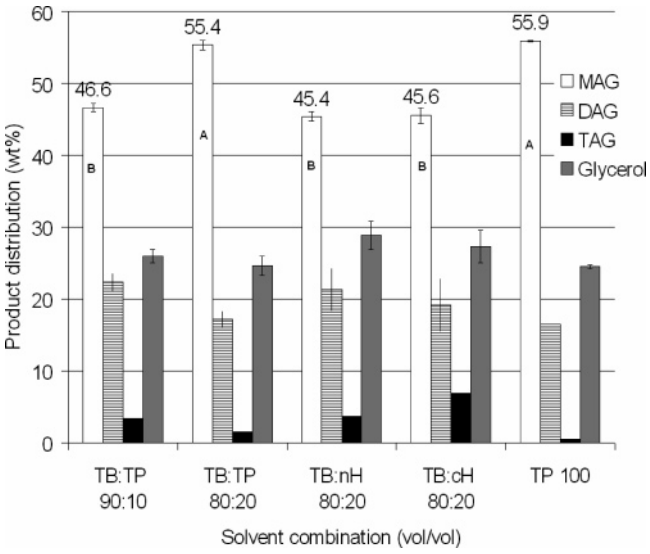


Figure 2. Measured product distribution (weight percent) after continuous glycerolysis in different binary solvent systems. Reaction conditions: glycerol to oil ratio, 5:1 mol/mol; temperature, 40 °C; solvent dosage, 20 mL/10 g of oil; reaction weight time, 31 min; and flow, 0.6 mL/min. Statistically significant different samples are represented by A and B. Error bars represent the standard deviation based on triple determinations.

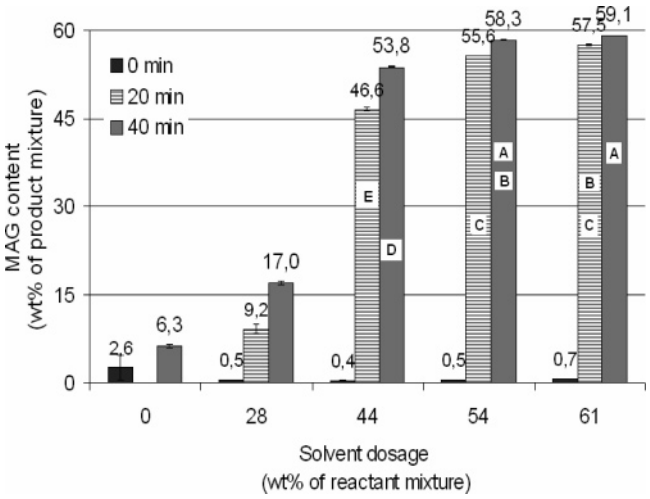


Figure 3. MAG content after continuous glycerolysis in varied dosages of a binary mixture of TB:TP 80:20 (volume percent) at reaction weight times of 20 and 40 min. The solvent-free system was added 5 wt % DimodanHR (high-purity MAG). Reaction conditions: glycerol to oil ratio, 3:1 mol/mol (apart from solvent-free system with molar ratio of 5); temperature, 40 °C; and flow, 0.47 or 0.93 mL/min. A–E indicate statistically significant different samples. Error bars represent the standard deviation based on double determinations.

An increased solvent dosage from 44 to 54% significantly enhanced the MAG content by 9 and 5% for reaction times of 20 and 40 min, respectively. In contrast, no significant effect on the MAG content was achieved with increased solvent dosage from 54 to 61%. A significant effect of the reaction time was obtained independent of the solvent dosage. A prolonged reaction time from 20 to 40 min enhanced the MAG content by 2−8% depending on the solvent dosage from 28 to 61%.

To identify the most optimal reaction time and solvent dosage, the MAG content per space−time was calculated, shown in **Table 2**. From **Table 2** it can be seen that a reaction time of 20 min and a solvent dosage of 44 or 54% yielded similar and highest possible space−time MAG contents. Prolonged reaction time and increased solvent dosage just lowers the MAG content per space−time, making these conditions less attractive. A solvent dosage of 54 wt % is believed to be most attractive to obtain the highest possible MAG content in the product mixture, whereas 44% solvent has the benefit of lower solvent consumption. Subsequently, purification is required for both reaction systems to obtain high-purity MAG products. Hence, whether varied MAG content and solvent dosages influence the purification process differently should be considered to identify the most attractive setup. Anyhow, a required solvent amount of 44−54 wt % is regarded as a relatively high dosage, which emphasizes the importance of recycle considerations to make the system industrially feasible/sustainable.

Table 2. MAG Content per Space Time (Weight Percent/Minute) after Glycerolysis in Varied Dosages of the Binary TB:TP 80:20% Mixture and Different Reaction Times Calculated as Weight Percent MAG Content Withdrawn Solvent Amount and Divided by the Reaction Time (Results Are Based on Double Determinations)

solvent dosage (wt %)	reaction time 20 min	reaction time 40 min
28	0.33	0.31
44	1.30	0.75
54	1.28	0.67
61	1.12	0.58

Phase Split Behavior. Glycerol and oil are immiscible. Polarity differences can help explain this finding: Glycerol has a very polar structure with three hydroxyl groups, indicated by a log *P* value of −1.76. Oil has a very nonpolar structure indicated by high log *P* values of 7.64 and 7.05 for oleic (C18:1) and linoleic acids (C18:2), respectively (*17*). Pure triolein and pure glycerol have been predicted to be completely soluble in TB at temperatures above 78 °C and in TP above 28 °C (*6*).

Table 3. Compound Distribution in Upper and Lower Phase of a Reaction Mixture of Glycerol, Oil, and Binary TB:TP 80:20% Mixture at Different Temperatures (Based on Double Determinations)

			distribution measured		
temperature (°C)	compound	weighted out (wt %)	upper phase (wt %)	lower phase (wt %)	total (wt %)
40	oil	36.0	34.6	1.7	36.3
	glycerol	13.8	1.4	15.4	16.8
	solvent	50.2	43.4	3.5	46.9
	total	100	79.4	20.6	100
75	oil	36.0	40.5		40.5
	glycerol	13.9	1.2	12.3	13.5
	solvent	50.1	44.6	1.4	46.0
	total	100	86.3	13.7	100

From this prediction, it was assumed that a binary mixture of TB:TP would result in a homogeneous reaction mixture somewhere in the temperature ranges from 40 to 100 °C (*6*). To verify this assumption, the miscibility behavior at temperatures from 40 to 100 °C was experimentally observed. In the solvent system, a two-phase system, with glycerol- and oil-rich phases, was surprisingly observed regardless of the temperature tested (40, 60, 75, 80, or 100 °C). A phase split occurred immediately after the stirrer was stopped, even though all of the mixtures seemed to be nicely mixed during stirring. Thus, contrary to our expectations, it was not possible to experimentally validate an upper critical solution temperature.

Glycerol and sunflower oil were mixed separately with TB:TP 80:20 vol % to test whether the phase split was caused by immiscibility of either glycerol or oil in the binary solvent mixture. Results showed that glycerol as well as sunflower oil, in pure form, was completely miscible in the binary solvent mixture even at relatively low temperatures of 20 °C. This confirms the predicted ability of the binary solvent mixture to dissolve polar as well as nonpolar compounds.

However, the observed phase split in the reaction mixture suggests a cosolute effect or indicates that the solvent mixture prefers one compound over the other when brought together. Thus, a closer investigation of the solvent and compound distribution in each phase was performed. Results are shown in **Table 3**. Measured values deviated by only <5% from amounts weighted out, which is ascribed to inaccuracies of the method. A clear separation of oil and glycerol in an upper and a lower phase was achieved. In the upper phase, the less dense oil accounted for 96−97 wt %, whereas the denser glycerol accounted for only <4 wt %. In the lower phase, the opposite tendency was obtained with 90−100 wt % of the dense glycerol and <10 wt % of the oil situated. This corresponds to oil:glycerol mole fractions of 72−78:22−28% in the upper phase and oil:glycerol mole fractions in the lower phase of 0−1:99−100%. The solvent distribution with >92% of the solvent situated in the upper oily phase and <3.5% in the lower glycerol phase clearly indicates that the solvent mixture has greater preference for oil compared to glycerol.

This obvious phase split behavior does not suggest particularly improved reactant miscibility in the solvent system compared to the solvent-free system. This suggests that the enhanced reaction efficiency in the solvent system, shown in **Figure 3**, also relates to aspects other than the improved system homogeneity. The activating effect of the solvent is believed to be due to sufficiently changed polarity of the system and reduced viscosity of the reactant mixture. In the presence of an organic medium a better emulsion of glycerol and oil with greater surface area accessible for the enzyme is most likely formed. It is also plausible that changes in the polarity prevent the adherence of glycerol to the enzyme, thereby avoiding a restricted contact between oil and enzyme. The solvent's capability to access the active site of the enzyme most likely favors the enzyme activity, too (*18*). Reduced viscosity of the reaction mixture makes the flow and thereby the access/contact to the enzyme easier with reduced mass transfer limitations. However, it should be noted that an inhomogeneous system still has the risk of mass transfer limitations due to inconsistent flow of the reactants. Finally, it should be noted that the phase split behavior was observed in the reaction mixture only. The conversion of TAG and glycerol to more amphiphilic MAG and DAG molecules, which have emulsifying properties, is believed to be among the reasons for a homogeneous product mixture.

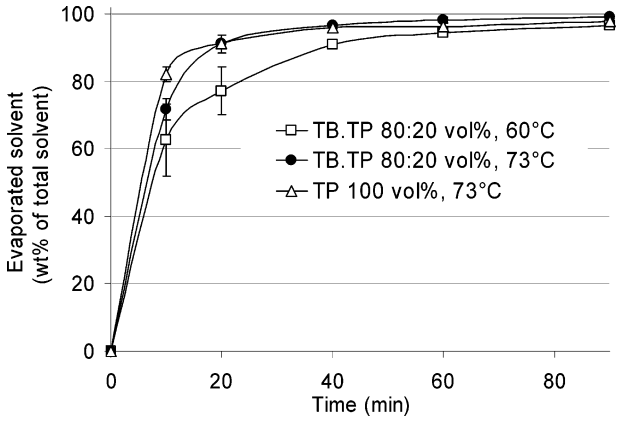


Figure 4. Time course for solvent removal during rotation evaporation under vacuum at 60 and 73 °C. Reaction mixture consisted of 10 g of oil mixed with 8.7 g of glycerol (glycerol to oil molar ratio of 8) and 20 mL of solvent of either 100% TP or a TB:TP 80:20% mixture. Error bars represent the standard deviation based on double determinations.

Solvent Removal by Rotation Evaporation. The ability to remove a TB:TP mixture and pure TP from a product mixture after glycerolysis was tested by rotation evaporation under vacuum at 60 and 73 °C. The results are given in **Figure 4**. Of the TB:TP 80:20 vol % mixture, 97 wt % was evaporated at 60 °C, whereas 99 wt % was evaporated at 73 °C. In comparison, 98 wt % of the pure TP was removed at 73 °C (**Figure 4**). The condensation processing was successfully conducted with no indication of crystallization in the equipment. Thus, the solvent removal was found to be fairly easy, and implementation of a more careful temperature and pressure control is expected to overcome the problems with the minor leftover in the product mixture.

The possibility of complete solvent removal at more controlled conditions was tested by conducting purification processing at industrial pilot plant facilities at DANISCO A/S. A product mixture obtained from continuous enzymatic glycerolysis reaction in a pure TP system (MAG, DAG, excess glycerol, and TAG and 50 wt % TP) was used for stripping and distillation processing. After this purification process, no solvent was left in the remaining product mixture. This verifies that solvent can be relatively easily removed from the product mixture after glycerolysis by currently used industrial purification techniques.

In conclusion, the TB:TP 80:20 vol % was shown to be a competitive alternative organic medium to pure TB or TP for efficient continuous enzymatic glycerolysis. The binary mixture

ensures high MAG formation in a short time, reasonable solvent price, and easy handling during distillation/condensation processing. A relatively high solvent dosage of minimum 44% was found to be necessary to achieve MAG yields attractive to industrial applications. However, reusability of the solvent and fast continuous operation is believed to overcome some of the problems with lowered space−time product yield. Even though solvents represent extra challenges from a safety point of view, the benefits of the solvents should by far exceed the drawbacks. Enhanced enzyme activity with only a very short required reaction time of 20 min makes the solvent system very attractive to industrial facilities compared to the much slower and more inefficient solvent-free system. Thus, implementation of the enzymatic glycerolysis in a binary mixture of TB:TP 80:20 vol % into industrial plants is believed to be the future production method for selected high-value MAG products for which a gentle technology at ambient temperatures is required.

ABBREVIATIONS USED

BP, boiling point; cH, cyclohexane; DAG, diacylglycerol; MAG, monoacylglycerol; MP, melting point; nH, *n*-hexane; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; TB, *tert*-butanol; TP, *tert*-pentanol.

SAFETY

As described under Results and Discussion highly flammable and harmful risks are related to hexane and TB as well as TP (*12*−*15*). According to European standards the maximum allowable exposure limits for the four solvents are *tert*-butanol, 150 mg/m^3, 50 ppm; *tert*-pentanol, 360 mg/m^3, 100 ppm; cyclohexane, 172 mg/m^3, 50 ppm; and *n*-hexane, 90 mg/m^3, 25 ppm (*12*−*15*). The inhaled vapor solvents can be accumulated in tissues with high lipid content (e.g., nerves, brain, bone marrow, adipose tissue, liver, and kidneys) and cause damage to the cells. Direct solvent−skin contact can lead to dissolving of the natural fatty layer, causing cracking of the skin with enhanced risk of infections or direct inflammations and blistering (*13*). Thus, special precautionary handling procedures should be taken when working with the solvents or solvent-containing preparations. Good ventilation is required, and gloves should be worn (*12*, *13*).

ACKNOWLEDGMENT

Technical assistance from Bodil Alrø, Pouel Andersen, and Sally Bording Pedersen, Danisco A/S, Brabrand, Denmark, is gratefully acknowledged.

LITERATURE CITED

(1) Kaewthong, W.; Kittikun, A.-H. Glycerolysis of palm olein by immobilized lipase PS in organic solvents. *Enzyme Microb. Technol.* **2004**, *35*, 218−222.

(2) Bornscheuer, U. T. Lipase-catalyzed syntheses of monoacylglycerols. *Enzyme Microb. Technol.* **1995**, *17*, 578−586.

(3) Bellot, J. C.; Choisnard, L.; Castillo, E.; Marty, A. Combining solvent engineering and thermodynamic modeling to enhance selectivity during monoglyceride synthesis by lipase-catalyzed esterification. *Enzyme Microb. Technol.* **2001**, *28* (4−5), 362−369.

(4) Min, D. B. Lipid oxidation of edible oil. In *Food Lipids*; Akoh, C., Min, D. B., Eds.; Dekker: New York, 1998; pp 283−296.

(5) Damstrup, M. L.; Jensen, T.; Sparsø, F. V.; Kiil, S. Z.; Jensen, A. D.; Xu, X. Production of heat sensitive monoacylglycerols by enzymatic glycerolysis in *tert*-pentanol: process optimization by response surface methodology. *J. Am. Oil Chem. Soc.* **2006**, *83* (1), 27−33.

(6) Damstrup, M. L.; Jensen, T.; Sparsø, F. V.; Kiil, S. Z.; Jensen, A. D.; Xu, X. Solvent optimization for effcient enzymatic monoacylglycerol production nased on a glycerolysis reaction. *J. Am. Oil Chem. Soc.* **2005**, *82* (8), 559−564.

(7) Yang, T.; Rebsdorf, M.; Engelrud, U.; Xu, X. Enzymatic production of monoacylglycerols containing polyunsaturated fatty acids through an efficient glycerolysis system. *J. Agric. Food Chem.* **2005**, *53*, 1475−1481.

(8) Hansen, H. K.; Rasmussen, P.; Fredenslund, A.; Schiller, M.; Gmehling, J. Vapor−liquid equilibria by UNIFAC group contribution. 5. Revision and extension. *Ind. Eng. Chem. Res.* **1991**, *30*, 2352−2355.

(9) Lide, D. R. Physical properties of organic compounds. In *Handbook of Chemistry and Physics*, 85th ed.; Lide, D. R., Ed.; CRC Press: Boca Raton, FL, 2004−2005; Section 3.

(10) Govindaswamy, S.; Andiappan, A.; Lakshmanan, S. Vapor−liquid equilibriums of the binary and ternary systems containing *n*-hexane (1)−benzene (2)-*tert*-butyl alcohol (3) at 760 mmHg pressure. *J. Chem. Eng. Data.* **1977**, *22*, 264−269.

(11) Reddy, M. S.; Rao, C. V. Vapour−liquid equilibria: benzene−*tert*-butanol and cyclohexane−*tert*-butanol systems. *Indian J. Technol.* **1965**, *3*, 45−48.

(12) Kemikaliebrugsanvisning for cyclo-hexane, *tert*-butanol, *n*-hexane og *tert*-pentylalkohol; www.kemibrug.dk/KBA (accessed Aug 17, 2005).

(13) Stoye, D. Solvents. In *Ullmann's Encyclopedia of Industrial Chemistry*, 7th ed.; Wiley-VCH Verlag: Weinheim, Germany, 2006; Section Solvents.

(14) Hahn, H.-D.; Dämbkes, G.; Rupprich, N. Butanols. In *Ullmann's Encyclopedia of Industrial Chemistry*, 7th ed.; Wiley-VCH Verlag: Weinheim, Germany, 2006; Section Butanols.

(15) Lappe, P.; Hofmann, T. Pentanols. In *Ullmann's Encyclopedia of Industrial Chemistry*, 7th ed.; Wiley-VCH Verlag: Weinheim, Germany, 2006; Section Pentanols.

(16) Food and Nutrition Board of the Institute of Medicine. General tests and assays. In *Food Chemicals Codex*, 5th ed.; Institute of Medicine of the National Academies; National Academy Press: Washington, DC, 2004; pp 827−962.

(17) Lide, D. R. Octanol−water partition coefficients. In *Handbook of Chemistry and Physics*, 85th ed.; Lide, D. R., Ed.; CRC Press: Boca Raton, FL, 2004−2005; Section 16.

(18) Martinelle, M.; Holmquist, M.; Hult, K. On the interfacial activation of *Candida antartica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochim. Biophys. Acta* **1995**, *1258*, 272−276.

Received for review May 15, 2006. Revised manuscript received July 19, 2006. Accepted July 28, 2006. Funding and support by DANISCO A/S, Brabrand, Denmark, is appreciated.

JF061365R

Paper IV

Damstrup, M.L., Kiil, S., Jensen, A.D., Sparsø, F.V., and Xu, X.

Process development of Continuous Glycerolysis in an Immobilized Enzyme-Packed Reactor for Industrial Monoacylglycerol Production.

J. Agric. Food Chem. 2007, 55 (19), 7786-7792.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Process Development of Continuous Glycerolysis in an Immobilized Enzyme-Packed Reactor for Industrial Monoacylglycerol Production

MARIANNE LINDE DAMSTRUP,*,† SØREN KIIL,‡ ANKER DEGN JENSEN,‡ FLEMMING VANG SPARSØ,§ AND XUEBING XU†

BioCentrum and Department of Chemical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, and Danisco A/S, DK-8220 Brabrand, Denmark

Continuous and easily operated glycerolysis was studied in different lipase-packed columns to evaluate the most potential process set-ups for industrial monoacylglycerol (MAG) production. Practical design-related issues such as enzyme-filling degree, required reaction time, mass transfer investigations, and capacity and stability of the enzyme were evaluated. A commercially available immobilized *Candida antarctica* lipase B was used to catalyze the glycerolysis reaction between glycerol and sunflower oil dissolved in a binary *tert*-butanol:*tert*-pentanol medium. Considering easy handling of the enzyme and measured expansion when wetted with a reaction mixture, a filling degree of 52 vol % dry enzymes particles per column volume seemed appropriate. Twenty minutes was required to reach equilibrium conditions with a MAG content of 50–55 wt %. Only insignificant indications of mass transfer limitations were observed. Hence, the commercial lipase seemed adequate to use in its available particle size distribution ranging from 300 to 900 μm. A column length-to-diameter ratio of less than 25 did not interfere with the transfer of the fluid mixture through the column. Under the tested conditions, the enzyme could be active for approximately 92 days before enzyme renewal was needed. This corresponds to a very high enzyme capacity with approximately 2000 L pure MAG produced per kg enzyme.

KEYWORDS: ***Candida antarctica*** **lipase B; continuous glycerolysis; enzyme capacity and stability; mass transfer limitations; monoacylglycerols; packed bed reactor**

INTRODUCTION

Lipase-catalyzed glycerolysis processing is of industrial interest since it can be carried out at ambient temperatures and atmospheric pressures (*1–3*). The gentle enzyme technology thereby offers the possibility of large-scale production of heat-sensitive mono- and diacylglycerols (MAG and DAG) with polyunsaturated fatty acid (PUFA) profiles. Well-preserved unsaturated MAG–DAG products, especially purified MAGs, can serve as nutritionally improved food additives and biocompatible emulsifiers and therefore have great potential for new applications in functional foods, pharmaceutical products, etc. (*2–4*).

The use of packed bed reactors (PBRs) is a common strategy to facilitate lipase-catalyzed glycerolysis and other fat interesterification reactions in a continuous and relatively simple way. Various investigators have confirmed the benefits of PBR for lipid modification (*1, 5–8*).

Previously, glycerolysis conducted in a column packed with immobilized lipase was highly potential to ensure a high MAG formation in a short time (*8, 9*). The high efficiency obtained in this system can be attributed to several parameters described below. Glycerol and oil mixing in a binary tertiary alcohol medium reduce the high reactant viscosity and promote improved homogeneity during reactor transit. The solvent acts as an inert carrier material for the reactants to the active site of the enzyme and thus enhances the enzyme activity in the microaqueous systems (*9, 10*). The high-density loading of immobilized enzymes in the PBR facilitates the enzyme contact to the reactant mixture. In addition, the use of lipase bound to a solid carrier material makes separation from product mixture easy and thus facilitates the enzyme reutilization (*4, 11*).

In spite of the improved reactant homogeneity in the solvent glycerolysis system, a heterogeneous reactant mixture with a glycerol-in-oil emulsion occurs (*9*). Hence, the movement of material from phase to phase as well as through the catalyst pores becomes important since it can influence the performance of the immobilized enzyme reactor. Mass transfer investigations of different-sized enzyme particles and varied flow rates, as well as enzyme reactor performance, have been analyzed for different

* To whom correspondence should be addressed. Tel: +45 4525 2636. E-mail: mali@biocentrum.dtu.dk.
† BioCentrum.
‡ Technical University of Denmark.
§ Danisco A/S.

10.1021/jf063366p CCC: $37.00
Published on Web 08/23/2007

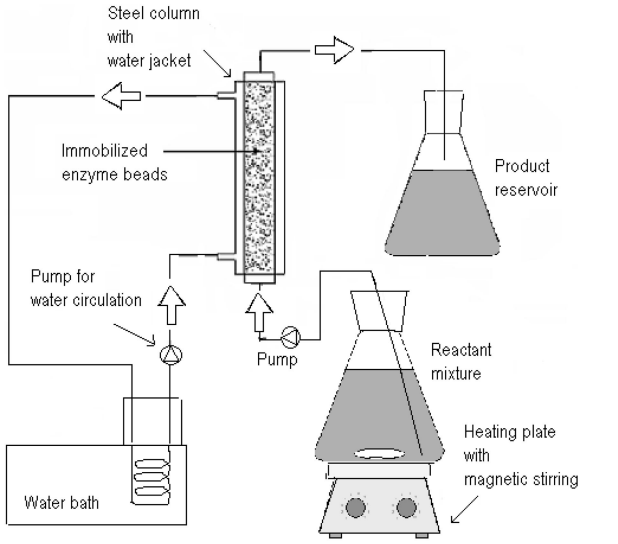


Figure 1. Illustration of the process set-up used for lipase-catalyzed glycerolysis in an enzyme-filled column.

fat interesterification reactions (*1, 5–8, 12*). However, to our knowledge, the available literature lacks investigations that combine these aspects for the glycerolysis process.

The costs of immobilized enzymes are generally high as compared to the costs of traditional chemical catalysts (*4*). Hence, longer use of the enzyme is essential to overcome some of the drawbacks of the increased expenses. Thus, evaluation of the enzyme capacity is crucial to rationalize the performance and operation efficiency.

The aim of this study was to examine the most appropriate process design for an efficient and simple glycerolysis process in an immobilized lipase-packed column reactor for industrial utilization. Practical design-related issues such as required reaction time, enzyme capacity, expansion of the enzyme during wetting, and the effect of different column length-to-diameter ratios, fluid velocities, and particle sizes of the enzymes were evaluated. These investigations were applied to reveal which basic features need to be considered to obtain an industrially beneficial procedure.

MATERIALS AND METHODS

Materials. Sunflower oil containing 97.1% triacylglycerol (TAG), 2.5% DAG, and 0.4% MAG was provided by AarhusKarlshamn AB (Aarhus, Denmark). The predominant fatty acids of the oil were C16:0, 6.7; C18:0, 3.7; C18:1, 26.3; and C18:2, 61.2 (wt %). Novozymes A/S (Bagsværd, Denmark) supplied Novozym435, a commercially available immobilized lipase from *Candida antarctica* lipase B (CALB). The lipase was immobilized by physical adsorption onto an acrylic type macroporous support based on a hydrophobic matrix of polymethyl methacrylate (PMMA) (Lewatit VP OC 1600). Characteristics of the porous spherical beads were as follows: bulk density, 430 kg/m^3; particle diameter, 300–900 μm; surface area, 80–150 m^2/g; and pore diameter, 140–170 10^{-10} m (*13, 14*). Glycerol with a purity of 99.5% w/w was purchased from VWR International Ltd. (Albertslund, Denmark). *tert*-Pentanol (TP) (2-methyl-2-butanol) with a purity of 99% and *tert*-butanol (TB) (2-methyl-2-pentanol) with purity of 99.7% were purchased from Sigma-Aldrich (Brøndby, Denmark).

Continuous Glycerolysis in Enzyme-Packed Column. The process set-up is illustrated in **Figure 1**. Reactant mixtures of sunflower oil, glycerol, and solvent were prepared in a glycerol-to-oil molar ratio of 4–5 and preheated to 50 °C under magnetic stirring. Each mixture, maintained at the set temperature, was pumped upwards through a column by a pump (FMI Laboratory Pump QG150, Micro Laboratory AARHUS A/S, Højbjerg, Denmark). The column consisted of a jacketed steel column packed with the dry enzyme as it was obtained. Unless otherwise stated, the column dimensions were as follows: 20 cm in length and 1.5 and 3.8 cm in inner and outer diameter, respectively. The column temperature was set to 40 °C, maintained by a water bath circulation. Initially, the enzyme bed was wetted and air was removed from the system by running reactant mixture through the column, equivalent to at least four times the reactor volume. To easily implement data into different enzyme dosages, reactor sizes, or flow rates, the following reaction time was used (*15*)

$$\tau = \frac{W}{\nu_0 \times \rho} \quad (1)$$

where τ is the reaction time (min), W is the weight of catalyst pellets on dry matter basis (g), ρ is the bulk density of the dry enzyme g/cm^3, and ν_0 is the volumetric flow rate of the reactant mixture (cm^3/min). Sampling was not started before flow through the enzyme bed equal to four times of the reaction time (τ) was reached. Two samples were hereafter taken and stored at −20 °C prior to analysis by gas chromatography (GC) as described elsewhere (*2*). Results are based on double determinations and are expressed as weight percentages of the product mixture based on glycerol (Gly), MAG, DAG, and TAG, free fatty acid (FFA), and fatty acid ester (FAE) contents.

Screening of Varied Enzyme Loadings in PBR. Glycerolysis with 4, 8, and 12 g of dry enzyme filled in the column was carried out. A reactant mixture of 52.7 wt % TB:TP 80:20 v/v, 33.3 wt % sunflower oil, and 14.0 wt % Gly was used. Different flow rates in the range from 0.24 to 1.9 mL/min were adjusted based on the enzyme amount (4, 8, and 12 g) to obtain reaction times of 5, 10, 15, 20, 30, and 40 min in the column. Down flow was used in this set-up to minimize the risk of back-mixing in the only partially enzyme-filled column.

Measurement of the Expansion Degree during Lipase Wetting. A reactant mixture consisting of 52.7 wt % TB:TP 80:20 v/v, 14.0 wt % Gly, and 33.3 wt % sunflower oil was mixed in a conic flask with magnetic stirring. Exactly 100 mL of the reactant mixture was filled in a measuring cylinder. Ten milliliters of dry immobilized lipase beads was carefully added to the measuring cylinder containing the reactant mixture. After 0.5, 1, and 24 h, the volume of the wetted bead "layer" was measured. The expansion factor was then calculated as the volume of wetted enzyme beads divided by the volume of the dry enzyme beads. Results are expressed as average values of triple determinations.

Screening of Different Reaction Times in PBR. Glycerolysis with 8 g of dry enzyme filled in the column was conducted at different reaction times. A reactant mixture consisting of 51.4 wt % TP, 31.8 wt % sunflower oil, and 16.8 wt % Gly was used. Different flow rates in the range from 0.47 to 1.24 mL/min were adjusted based on the enzyme amount to obtain reaction times of 10, 15, 20, 25, 35, and 40 min in the column.

Operation Stability/Capacity of the Lipase in PBR. Glycerolysis with 8 g of dry enzyme filled in the column was conducted under two conditions. (A) In a pure TP system for 42 days. Here, mixtures of 51.4 wt % TP, 16.8 wt % Gly, and 31.9 wt % sunflower oil were pumped from 5 L flasks through the column at a flow rate of 0.6 mL/min to obtain a reaction time of 30 min. (B) In a binary TB:TP system for 48 days. Here, mixtures of 50.0 wt % TB:TP 80:20 v/v, 17.2 wt % Gly, and 32.8 wt % sunflower oil were pumped through the column at a flow rate of 0.95 mL/min to obtain a reaction time of 20 min.

Glycerolysis with Different Particle Sizes of Enzyme Fractions. Novozym435 was carefully separated by a 500 μm sieve into two fractions. The average particle sizes of the two fractions were then measured with a Mastersizer 2000, Malvern Instruments (Leeds, United Kingdom). The PBR glycerolysis reaction was then performed with 4 g of each enzyme fraction and in the downward flow. A reactant mixture consisting of 52.8 wt % TB:TP 80:20 v/v, 33.3 wt % sunflower oil, and 13.9 wt % Gly was used. Different flow rates in the range from 0.24 to 1.9 mL/min were adjusted based on the enzyme amount to obtain a reaction time of 5, 10, 15, 20, 30, and 40 min in the column. Double determinations were made of samples withdrawn after running four and seven times of the reaction time. The experiments were repeated at reaction times of 20 and 40 min to evaluate the inaccuracy of the method. The reaction rates (r_A) for the two enzyme fractions were calculated as the change in mole MAG per unit time (a molar weight of MAG of 355 g/mol was used for the calculation).

Table 1. Column Dimensions, Enzyme Loadings, Flow Rates, and Reaction Times Used for Glycerolysis in PBR with Different Set-ups as Compared to Calculated Fluid Velocity, Reynolds Number, and Measured MAG Content after Reaction

column dimension			reaction parameters			calculated/measured		
diameter (d) (mm)	length (l) (mm)	volume (V) (cm^3)	enzyme loading (kg/m^3)	reaction time (τ) (min)	flow (mL/min)	fluid velocity (v) (m/s)	MAG content (wt %)	reaction rate (r_A)
34	500	454.0	220	20	11.60	8.4×10^{-5}	55.6 ± 0.3^a	7.84
21	500	173.2	220	20	4.60	2.2×10^{-4}	52.9 ± 0.4^a	7.46
21	400	138.5	220	20	3.50	1.7×10^{-4}	52.5 ± 0.7^a	7.40
21	200	69.3	220	20	1.80	8.7×10^{-5}	53.9 ± 0.4^a	7.60
15	200	35.3	220	20	0.95	9.0×10^{-5}	50.2 ± 1.5^a	7.08
15	200	35.3	227	20	0.92	8.7×10^{-5}	51.2 ± 2.1^b	7.22
15	200	35.3	227	27	0.69	6.5×10^{-5}	54.0 ± 0.3^b	5.64
15	200	35.3	227	34	0.55	5.2×10^{-5}	56.6 ± 0.2^b	4.69
15	200	35.3	227	41	0.46	4.3×10^{-5}	56.6 ± 0.1^b	3.89
21	200	69.3	173	10	2.80	1.4×10^{-4}	44.8 ± 2.0^b	12.63
21	200	69.3	173	20	1.40	6.7×10^{-5}	50.8 ± 2.1^c	7.16
21	200	69.3	173	31	0.90	4.3×10^{-5}	52.0 ± 1.2^b	4.73
15	400	70.7	173	10	2.85	2.7×10^{-4}	44.7 ± 0.3^b	12.61
15	400	70.7	173	20	1.40	1.3×10^{-4}	49.3 ± 5.3^c	6.95
15	400	70.7	173	30	0.95	9.0×10^{-5}	54.2 ± 1.0^c	5.10

[a] Average values of triple determinations ± standard deviation. [b] Average values of double determinations ± standard deviation. [c] Average values of four determinations (two repeated experiments, each with double determinations) ± standard deviation.

Reactions in PBR with Different Dimensions/Flow Rates. Various flow rates from 0.95 to 11.60 mL/min were tested in five different columns at a constant reaction time of 20 min and constant enzyme loading of 220 kg/m^3 in the column. Lower flow rates from 0.46 to 0.92 mL/min were tested in the smallest column at a constant enzyme loading of 227 kg/m^3. Reaction times of 10, 20, and 30 min were examined in columns with different length-to-diameter ratios but with constant enzyme loading of 173 kg/m^3. Used column dimensions, enzyme loadings, flow rates, and reaction times for each of these set-ups are summarized in **Table 1**. The used reactant mixtures were identical for all set-ups and consisted of 52.8 wt % TB: TP 80:20 v/v, 33.3 wt % sunflower oil, and 13.9 wt % Gly. To characterize the pipe flow through the enzyme particles in each column, calculation of the Reynolds number (Re) was performed as (*16*):

$$\mathrm{Re} = \frac{d_p \times v \times \rho}{\mu} \quad (2)$$

where d_p is the enzyme particle diameter set to 0.6 mm, v is the fluid velocity calculated as flow/cross area of the column, and ρ is the fluid density set to 900 kg/ m^3. μ is the fluid viscosity of the reactant mixture measured as 62 mPa s at 25 °C with Viscometer–KR140 (Research Equipment Ltd., London, United Kingdom). The flow was hereafter characterized as turbulent if Re ≥ 4000 or laminar if Re ≤ 2000 (*16*). The fluid moving past single particles was then evaluated by calculating the Sherwood number (Sh) as (*15*):

$$\mathrm{Sh} = \frac{k \times d_p}{D} = 2 + 0.6(\mathrm{Re})^{1/2}(\mathrm{Sc})^{1/3} \quad (3)$$

where k is the mass transfer coefficient and the Schmidt number (Sc) is defined as:

$$\mathrm{Sc} = \frac{\mu}{\rho \times D} \quad (4)$$

with the molecular diffusion coefficient (D) estimated to be 10^{-10} m^2/ s.

RESULTS AND DISCUSSION

Effect of Varied Enzyme Loadings in PBR. To determine the most appropriate filling degree of dry enzyme in the column, the effect of three loadings on the MAG formation was tested. Results are shown in **Figure 2**. A medium enzyme dosage of 227 kg dry enzyme/m^3 column, corresponding to 52% of the column volume being filled with dry enzyme beads, resulted in consistent results with increased MAG formation at the prolonged reaction time up to 40 min. A lower or higher enzyme load of 114 and 340 kg/m^3 column, corresponding to filling degrees of 26 and 77 vol %, respectively, led to more inconsistent results.

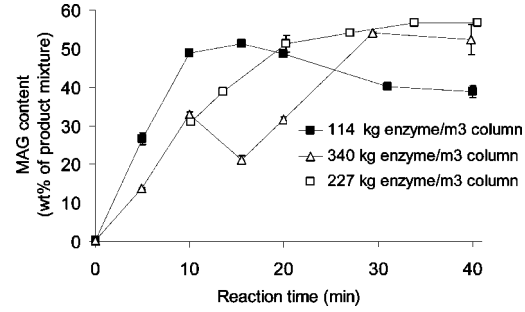


Figure 2. Measured MAG content after glycerolysis at different reaction times in a column filled with varying enzyme loadings (4, 8, and 12 g). Reaction conditions: 52.7 wt % binary TB:TP mixture, glycerol-to-oil molar ratio of 4, reaction temperature of 40 °C, and flow rates from 0.24 to 1.9 mL/min. Error bars represent standard deviations from double determinations.

At the low enzyme load of 114 kg dry enzyme/m^3column, the highest achieved MAG content of 51 wt % was obtained after 15 min of reaction and hereafter decreased. This surprising tendency with decreased MAG formation at the prolonged reaction time from 15 to 40 min indicates an unstable set-up. Thus, the low enzyme load somehow resulted in an unevenly reactant–enzyme contact, although downward flow was expected to prevent the risk of channel formation and thereby insufficient contact between reactants and enzyme. For the reaction with high enzyme load, the MAG contents also varied a lot at the different reaction times (**Figure 2**). At the high enzyme load of 340 kg enzyme/m^3column, it is plausible that the very tightly packed enzyme layer partly covered the active site of the enzyme. Hence, the reactants might be inaccessible to the active enzyme, thereby leading to reduced efficiency (*5*). Merely an overload of enzyme should be avoided to minimize problems with pressure drop and physical blocking of the column. Therefore, a medium level loading of approximate 227 kg dry enzyme/m^3 column, corresponding to a filling degree of 52 vol %, seemed most suitable for practical operations.

Table 2. Measured Expansion of the Dry Immobilized Lipase (Novozym435) after Wetting with Reactant Mixture for Different Times

time (h)	dry enzyme (mL)	wet enzyme (mL)	expansion (vol %[a])
0.5	10.3 ± 1.0	19.8 ± 2.0	191.9 ± 2.8
1	10.3 ± 1.0	19.8 ± 2.0	191.9 ± 2.8
24	10.3 ± 1.0	19.8 ± 2.0	191.9 ± 2.8

[a] Average values of triple determinations ± standard deviation.

Enzyme Expansion after Wetting with Reactant Mixture. Because the filling degree of 227 kg dry enzyme per m^3 column corresponds to only 52% of the column volume, enzyme swelling during wetting was indicated. The expansion of the immobilized lipase particles was therefore investigated. Results are given in **Table 2**. A quite pronounced expansion degree of 192 vol % of the enzyme–carrier complex was observed as the enzyme changed from dry to wet conditions (**Table 2**). This is in accordance with the literature where the tested enzyme carrier PMMA material swells in some organic solvents (*17, 18*). The observed expansion degree of 192 vol % agrees with a dry enzyme load of 227 kg /m^3 column being the right amount to achieve a total enzyme-filled column after being wetted and stabilized.

A simplified column packing is attractive for practical operations. A full enzyme column is most advantageous to control the flow conditions and optimally utilize the reactor volume. Furthermore, possible back-mixing is avoided and a more complex flow behavior of the fluids does not need to be taken into consideration. Our results show that the time did not influence the expansion degree of the enzyme (**Table 2**). This indicates that swelling saturation of the enzyme is ensured within 30 min. Thus, initial wetting of the dry enzyme bed can easily be done directly in the column in reasonable time before the actual glycerolysis reaction is started. Hence, it is found unnecessary to add glass beads or another filling material in the column to fix the enzyme bed.

Effect of Different Reaction Times. Different reaction times for continuous glycerolysis in the organic medium are evaluated by Yang et al. (*8*) who found a residence time of 30–40 min sufficient to reach maximum MAG yields (*8*). In the actual set-up, a reaction time defined by eq 1 is preferred, since it can easily be used for a solid-catalyzed reaction. To identify the most optimal reaction time based on this equation, reaction times from 10 to 40 min were evaluated.

The time courses are illustrated in **Figure 3**. In general, an increased MAG content was observed with prolonged reaction time reaching the maximum MAG content of 55 wt % after 40 min (**Figure 3**). However, only a minor MAG increase of 5 wt % was obtained as the reaction time was prolonged from 20 to 40 min. Therefore, a reaction time of 20 min should be sufficient to reach an acceptable high MAG formation in accordance with early findings (*9*).

Enzyme capacity or process productivity in terms of MAG content per volume per unit of time is naturally reduced with prolonged reaction time. Hence, the reaction time should be carefully considered to identify the most attractive set-up. The first 10 min led to a MAG content of 31 wt % as compared to only a 19 wt % MAG content increase at the second 10 min (**Figure 3**). A high content of impurities/unwanted side products in the product mixture in general complicates the purification process. Thus, we believe that 20 min will be most beneficial from a practical point of view. Still, a reaction time of 20 min is very short and definitely matches industrial requirements for a rapid and efficient reaction.

Operation Stability and Capacity of the Enzyme in PBR. Long-term continuous glycerolysis was conducted in two different solvent media to investigate the stability and process capacity performance of the lipase for MAG production. The achieved stability calculated with pure MAG produced per hour is illustrated in **Figure 4**, and other parameters characterizing the enzyme performance/productivity are summarized in **Table 3**. In general, the enzyme showed very good stability, capacity, and long half-lives in both solvent systems and maintained activity even after 1000 operation hours.

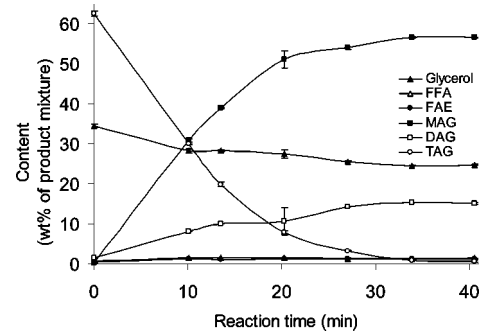


Figure 3. Measured product distribution after glycerolysis in an enzyme-filled column at varying reaction times. Reaction conditions: 51.4 wt % TP, glycerol-to-oil molar ratio of 5, reaction temperature of 40 °C, and flow rates from 0.47 to 1.24 mL/min. Error bars represent standard deviations from double determinations.

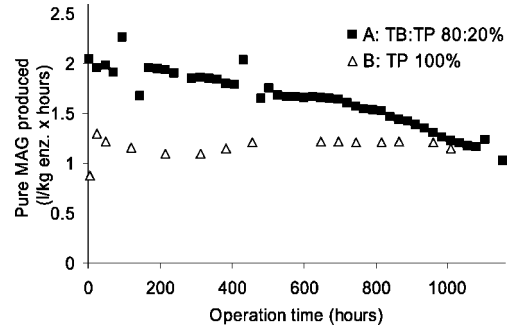


Figure 4. Time course for MAG production per hour after long-term continuous glycerolysis in (**A**) TB:TP 80:20 v/v system (50 wt %) and (**B**) pure TP system (51.4 wt %). Conditions for (**A**): a reaction time of 20 min, a flow rate of 0.95 mL/min, and 48 running days. In (**B**): a reaction time of 30 min, a flow rate of 0.60 mL/min, and 42 running days. Other reaction parameters: glycerol-to-oil molar ratio 5, 40 °C, and 8 g of enzyme.

A higher initial MAG production rate (MAG content/h) was observed in the TB:TP system than that in the TP system, which was most likely ascribed to variations in the reaction parameters (**Figure 4**). Hence, the 50% higher flow rate used in the TB:TP system agrees with the findings of initially 54% more MAG produced per hour. In both systems, the reaction was very fast and had reached equilibrium (50–55 wt %) without effects by the different flow rates.

A considerably more stable TP system was observed in contrast to a clear linear time-dependent decay in the TB:TP system (**Figure 4**). This resulted in remarkably different estimated residual activities of 55 and 90% for the TB:TP and the TP systems, respectively (**Table 3**). Comparison of MAG produced in the two systems at residual activity of 90% indicated a surprisingly higher capacity in the TP system with 25% more MAG produced even with the higher productivity per hour in the TB:TP system considered (**Table 3**). TP only differs from TB by one more carbon atom placed in the alcohol chain (*8*). This certainly leads to a slight difference of polarity, which might bring some difference of the enzyme activity. However, such effects were not noticed in early studies (*8*).

Table 3. Different Calculated Parameters Based on Results Obtained from Long-Term Continuous Glycerolysis in a TB:TP 80:20 v/v and a Pure TP System

parameter	unit	TB:TP system	TP system
operation time	h	1152	1008
amount used reactant mixture	L	65.7	36.3
total MAG produced[a]	L pure MAG/kg enzyme	1225	1100
final residual activity[b]	%	55	90
reactant exposure at 90% residual enzyme activity[c]	L reactant mixture /kg enzyme	3600	4500
total MAG produced at 90% residual enzyme activity[c]	L pure MAG/kg enzyme	890	1100

[a] Calculated as the area under the curves in **Figure 4** for the entire operation period. [b] Calculated as measured MAG content after ended operation time divided with initial MAG content (set to 100%). [c] The residual enzyme activity was based on the measured MAG content with initially MAG content set to 100%.

Typically, enzymes gradually get less active over time due to deactivation/inhibition. Both systems were exposed to identical temperature, similar operation time, same batch of enzyme, and substrate mixtures with the same source of solvent and Gly, as well as both running in laminar flows. Therefore, similar lipase-inhibiting effects were expected over time such as conformational changes, water deprivation from the enzyme, release of the carrier bonded to the enzyme, disruption in the reactant mixture, etc. (*19, 20*).

The high total reactant mixture exposure in the TP:TB system as compared to the TP system was the only difference that could have influence on the enzyme stability after all of the diagnoses. This indicates a certain exposure limit of the reactant mixture before a decrease in the enzyme activity occurs. Exposure limits of 28.7 and 36.3 L substrate/kg enzyme were determined for the TB:TP and TP systems, respectively, before a significant drop in the MAG production was detected.

In spite of the difference of stabilities in the two organic media (**Figure 4**), deactivation will certainly happen over time, explaining the need for catalyst replacement during the processing. Assuming that the enzyme activity in the TB:TP system follows the common first-order deactivation kinetics, the life time of the enzyme can be estimated from the following equation:

$$A = A_0 \times \exp^{-kd \times \tau} \quad (5)$$

where A and A_0 are enzyme activity at time τ and 0, respectively, and k_d is the deactivation constant. With a half-life set to 1200 h (based on estimation from **Figure 4**) and the lower limit for enzyme usage set to 25% residual activity, the enzyme life time is calculated to 2200 h (92 days). This corresponds to a total productivity roughly calculated as 2000 L pure MAG/kg enzyme. This makes the enzyme attractive for use in commercial plants and offers high economical potentiality for industrial implementation.

Effect of Different Particle Sizes of Enzyme. It has been established that the selection of support material and immobilization method is important for the reaction efficiency of enzymes (*5*). Previous experiments have already confirmed that the hydrophobic PMMA carrier material is beneficial as compared to many other carrier materials for lipase-catalyzed glycerolysis (*8, 21*). However, it is unclear how the particle size of this catalyst affects the reaction efficiency. To address this, pore diffusion resistance was evaluated by conducting continuous glycerolysis with different particle sizes of catalyst beads.

The time courses with average enzyme particle sizes of 388 and 548 μm are illustrated in **Figure 5**, and the calculated reaction rates are shown in **Table 4**. Some differences in the MAG content were observed (**Figure 5**). The initial reaction rate was 30% higher for the 5 min reaction performed with the smaller particles as compared to the larger particles (**Table 4**).

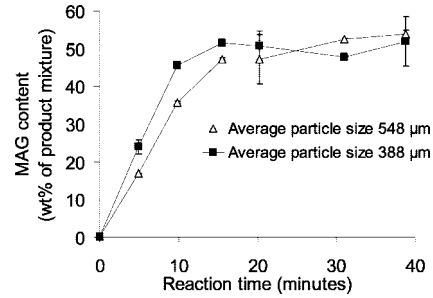


Figure 5. Measured MAG content after glycerolysis in a column filled with different-sized enzyme particles. Reaction conditions: 52.8 wt % binary TB:TP mixture, glycerol-to-oil molar ratio of 4, 40 °C, 4 g of enzyme, and flow rates from 0.24 to 1.9 mL/min. Error bars represent standard deviations.

Table 4. Calculated Reaction Rate (r_A) for MAG Formation Based on Glycerolysis Performed with Different Sizes of Enzyme Particles

time (min)	reaction rate (mmol MAG /min)[a] average particle 388 μm	average particle 548 μm	$r_{A388\mu m}/r_{A548\mu m}$
5	13.70	9.59	1.43
10	13.05	10.23	1.28
16	9.30	8.52	1.09
20	7.03	6.54	1.07
31	4.32	4.74	0.91
39	3.75	3.89	0.96

The difference of initial reaction rates indicates the existence of internal mass transfer limitations for the large particles used. Our observation is in accordance with the findings by Murty et al. for other lipase particles used in the continuous PBR (*1*).

However, the influence of the particle size was not significant in general (**Figure 5**). The reaction rate also converged as the reaction times were prolonged (**Table 4**). If 20 min is selected for the aimed reaction time as previously discussed, the difference of MAG content (**Figure 5**) as well as reaction rate (**Table 4**) is not significantly marginal. Therefore, differences between small and large particles are believed to be only minor in an overall consideration. Furthermore, the reduction of particle sizes of enzyme will increase the pressure drop in principle. This will certainly not be favorable for the process design. With these considerations in mind, internal mass transfer limitations with strong diffusion resistance through the porous particles are neglected. Thus, the lipase is believed to be adequate to use as it is with its commercially available size distribution ranging from 300 to 900 μm.

Effect of Different Flow Rates/Column Dimensions. Studies of oil hydrolysis in immobilized lipase PBR have shown that the reaction rate is affected by the linear fluid flow rate (*1*). Hence, external mass transfer limitations with poor transfer of the reactant mixture to the outer surface of the enzyme particles and poor product transport away from the particle

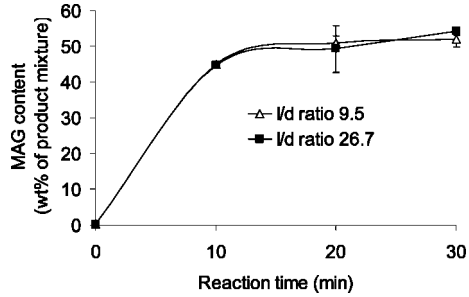


Figure 6. Measured MAG content after glycerolysis in columns with varied length-to-diameter (*l*/*d*) ratios. Reaction conditions: solvent dosage, 52.8 wt % TB:TP mixture; glycerol-to-oil molar ratio of 4; and temperature, 40 °C. In *l*/*d* 9.5: column diameter, 21 mm; column length, 200 mm; flow rates from 0.9 to 5.6 mL/min; and enzyme amount, 12.0 g. In *l*/*d* 26.7: column diameter, 15 mm; column length, 400 mm; flow rates from 0.9 to 5.8 mL/min; and 12.3 g of enzymes. Error bars represent standard deviations.

Table 5. Calculated Sherwood Number for Glycerolysis Performed in Two Columns with Different Length-to-Diameter Ratios

reaction time (min)	*l*/*d* ratio 9.5	*l*/*d* ratio 26.7	difference %
10	3.85	4.57	15.7
20	3.28	3.78	13.3
30	3.03	3.48	13.2

surface into the bulk phase are plausible in the actual set-up. In general, external mass transfer limitations are reduced in PBR by increased flow rates or reduced reactor length-to-diameter ratios, eventually leading to a higher linear flow rate (*5*). Thus, clarification of the effect of glycerolysis conducted with different flow rates and column dimensions was evaluated. The MAG formation and reaction rate under different conditions are summarized in **Table 1**. The MAG content achieved from glycerolysis in a long thin column as compared to a shorter thicker column is illustrated in **Figure 6**, and the corresponding calculated Sherwood numbers are shown in **Table 5**.

Very low Reynolds numbers of less than 100 were calculated for all experiments indicating a laminar flow behavior (Re < 2000). Hence, the flow seems to be dominated by viscous forces with, in theory, uniform nonturbulent flow in parallel layers with little mixing between layers (*16*). A certain degree of viscous forces being dominant for the flow dynamics is in good agreement with the high viscous oil and glycerol raw material although the presence of solvent indeed has reduced the viscosity of the reactant mixture (measured to be 62 mPa s at 25 °C).

At 20 min reaction, similar reaction rates were observed (approximately 7.3 ± 0.3 mmol/min), independent of the different fluid velocities tested (**Table 1**). This indicates that even low fluid velocities are not critical for the MAG formation and that the transfer of the reactant mixture to the enzyme is not a limiting factor for the process. The fluid velocities yielding laminar flow apparently caused an evenly distributed reaction mixture to the enzyme layer.

Almost identical time courses with similar MAG formations were observed for the two columns with different length-to-diameter ratios (**Figure 6**). Some differences were observed in the calculated Sherwood number with higher values in the long thin column as compared to the shorter thicker column (**Table 5**). Although this indicates a higher external mass transfer resistance in the long thin column as compared to the shorter thicker column, these differences are believed to be negligible. The reason for this is that a Sherwood number of the same order of magnitude for the two different columns can be treated as an insignificant difference. Hence, it seems unnecessary to consider alteration of the column reactor length-to-diameter ratios to dramatically improve the transfer of the fluid mixture through the column.

On the basis of our results, a scaled-up continuous glycerolysis process should not have considerably strong external mass transfer problems within the range of the length-to-diameter ratios used in this study. In a scaled-up reactor with increased dimensions, higher flow rates are used to maintain similar fluid velocity. Thus, it should be kept in mind that higher flow rates can increase the pressure drop and thereby the risk for pump malfunctions, enzyme bed compression, blockage, etc. (*8*). However, the set-up with laminar upward flow pattern and regularly shaped particles is believed beneficial for reducing pressure drop problems (*5*). In addition, calculated pressure drops of less than 1 bar per m/h for glycerolysis confirm minimized risk to encounter pressure drop problems in practical large-scale operations (*8*).

In conclusion, our results showed no foreseen difficulties in establishing an efficient and continuous glycerolysis reaction in an enzyme-packed reactor with respect to industrial operations. Simple dry enzyme packing of the column, no distinct mass transfer limitations, high capacity, and long-lasting activity of the enzyme makes the PBR set-up very suitable for scale-up processing without any obvious problems. Thus, the fulfillment of an easy and practical operation in combination with high reaction efficiency clearly demonstrates the great potential for future implementation of this process in industrial usage.

ABBREVIATIONS USED

CALB, *Candida antartica* lipase B; DAG, diacylglycerol; FAE, fatty acid esters; FFA, free fatty acids; GC, gas chromatography; Gly, glycerol; MAG, monoacylglycerol; PBR, packed bed reactor; PMMA, polymethyl methacrylate; PUFA, polyunsaturated fatty acids; TAG, triacylglycerols; TB, *tert*-butanol; and TP, *tert*-pentanol.

ACKNOWLEDGMENT

Technical assistance from Bodil Alrø, Danisco A/S, and Lilian Beenfeldt Holgersen, Department of Chemical Engineering-DTU, is gratefully acknowledged.

LITERATURE CITED

(1) Murty, V. R.; Bhat, J.; Muniswaran, P. K. A. Mass transfer effects in immobilized lipase packed bed reactor during hydrolysis of rice bran oil. *Chem. Biochem. Eng. Q.* **2004**, *18*, 177–182.

(2) Damstrup, M. L.; Jensen, T.; Sparsø, F. V.; Kiil, S. Z.; Jensen, A. D.; Xu, X. Solvent optimization for efficient enzymatic monoacylglycerol production based on a glycerolysis reaction. *J. Am. Oil Chem. Soc.* **2005**, *82*, 559–564.

(3) Damstrup, M. L.; Jensen, T.; Sparsø, F. V.; Kiil, S. Z.; Jensen, A. D.; Xu, X. Production of heat sensitive monoacylglycerols by enzymatic glycerolysis in tert-pentanol: process optimization by response surface methodology. *J. Am. Oil Chem. Soc.* **2006**, *83*, 27–33.

(4) Kaewthong, W.; Sirisansaneeyakul, S.; Prasertsan, P.; H-Kittikun, A. Continuous production of monoacylglycerols by glycerolysis of palm oil with immobilized lipase. *Process Biochem.* **2005**, *40*, 1525–1530.

(5) Ison, P.; Macrae, A. R.; Smith, C. G.; Bosley, J. Mass transfer effects in solvent-free fat interesterification reactions: Influences on catalyst design. *Biotechnol. Bioeng.* **1994**, *43*, 122–130.

(6) Garcia, H. S.; Yang, B.; Parkin, K. L Continuous reactor for enzymatic glycerolysis of butter oil in the absence of solvent. *Food Res. Int.* **1996**, *28*, 605–609.

(7) Xu, X.; Balchen, S.; Hoy, C. E.; Adler-Nissen, J. Production of specific-structured lipids by enzymatic interesterification in a pilot continuous enzyme bed reactor. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1573–1579.
(8) Yang, T.; Rebsdorf, M.; Engelrud, U.; Xu, X. Monoacylglycerol synthesis via enzymatic glycerolysis using a simple and efficient reaction system. *J. Food Lipids* **2005**, *12*, 299–312.
(9) Damstrup, M. L.; Abildskov, J.; Kiil, S.; Jensen, A. D.; Sparsø, F. V.; Xu, X. Evaluation of binary solvent mixtures for efficient monoacylglycerol production by continuous enzymatic glycerolysis. *J. Agric. Food Chem.* **2006**, *54*, 7113–7119.
(10) Zaks, A. Enzymes in organic solvents. In *Biocatalysis for Industry*; Dordick, J. S., Ed.; Plenum Press: New York, 1991, pp 161–180.
(11) Moreno, P. A. G.; Medina, A. R.; Rubio, F. C.; Paéz, B. C.; Cerdan, L. E.; Grima, E. M. Production of structured triacylglycerols in an immobilized lipase packed-bed reactor: Batch mode operation. *J. Chem. Technol. Biotechnol.* **2005**, *80*, 35–43.
(12) Murty, V. R. C.; Bhat, J.; Muniswaran, P. K. A. External mass transfer effects during the hydrolysis of rice bran oil in immobilized lipase packed bed reactor. *Chem. Biochem. Eng. Q.* **2005**, *19*, 57–61.
(13) Nakaoki, T.; Mei, Y.; Miller, L. M.; Kumar, A.; Kalra, B.; Miller, M. E.; Kirk, O.; Christensen, M.; Gross, R. A*Candida antarctica* lipase B catalyzed polymerization of lactones: Effects of immobilization matrices on polymerization kinetics and molecular weight. *Ind. Biotechnol.* **2005**, *1*, 126–134.
(14) Novozymes A/S. Novozym 435, Product Sheet, Oils & Fats/2001-06950-03.pdf. Novozymes A/S: Bagsværd, Denmark, 2001; pp 1–3.
(15) Levenspiel, O. Solid catalyzed reactions. In *Chemical Reaction Engineering*; Levenspiel, O., Ed.; John Wiley & Sons: New York, 1999; pp 376–426.
(16) Lide, D. R. Definition of scientific terms. In *Handbook of Chemistry and Physics*, 87th ed.; Lide, D. R., Ed.; CRC Press: Boca Raton, FL, 2006–2007; Section 2.
(17) Kaneda, I.; Vincent, B. Swelling behavior of PMMA-g-PEO microgel particles by organic solvents. *J. Colloid Interface Sci.* **2004**, *274*, 49–54.
(18) Ruzzu, A.; Matthis, B. Swelling of PMMA-structures in aqueous solutions and room temperature Ni-electroforming. *Microsyst. Technol.* **2002**, *8*, 116–119.
(19) Weete, J. D. Microbial lipases. In *Food Lipids*; Akoh, C. C.; Min, D. B., Eds.; Marcel Dekker: New York, 1998; pp 641–664.
(20) Hayes, D. G. Lipid modification in water-in-oil microemulsions. In *Enzymes in Lipid Modification*; Bornscheuer, U. T., Ed.; Wiley-VCH Verlag: Weinheim, Germany, 2000; pp 46–69.
(21) Kristensen, J. B.; Xu, X.; Mu, H. Diacylglycerol synthesis by enzymatic glycerolysis: Screening of commercially available lipases. *J. Am. Chem. Soc.* **2005**, *82*, 329–334.

Received for review November 21, 2006. Revised manuscript received June 27, 2007. Accepted July 9, 2007. Financial funding and support by Dansico A/S (Brabrand, Denmark) is appreciated.

JF063366P

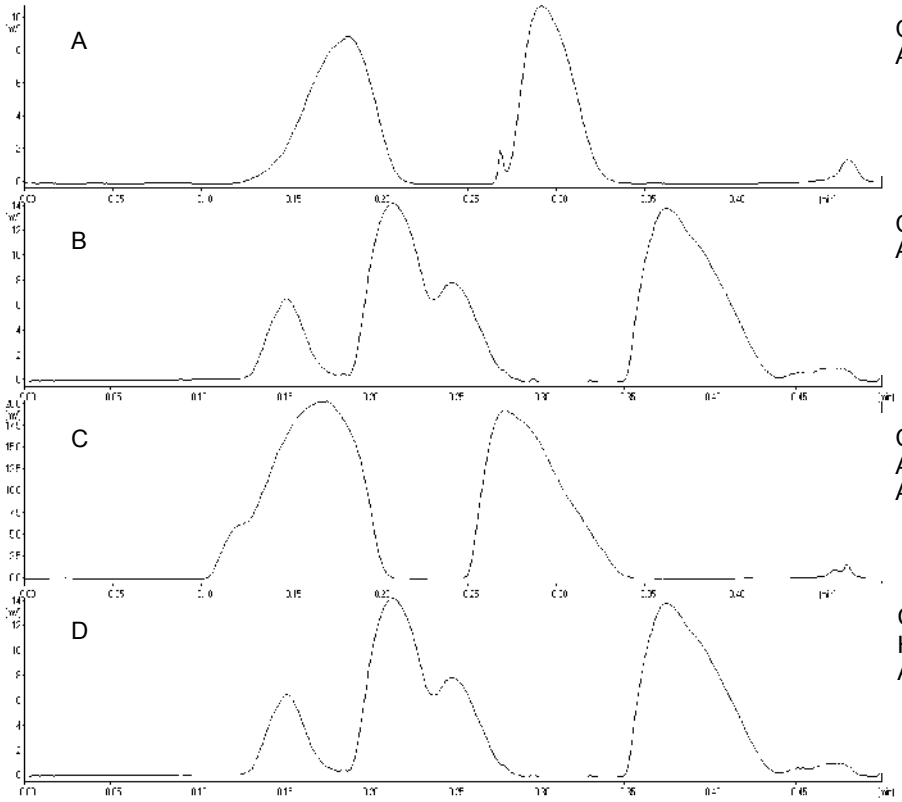
Appendix I

Description of TLC-FIDs applicability to measure compound distribution after enzymatic glycerolysis of vegetable oils and comparison to GC-FID analyses

Objective:
To optimize the composition of the solvent for the TAG; DAG and MAG separation on TLC-FID rods.
Evaluate the applicability of different standards to quantize MAG, DAG and TAG.
Investigate the usability of internal standards and response factors.
Clarify the accuracy of the TLC-FID method.
Compare results from sample measurement on TLC-FID and GC-FID.

Materials:
Pure standards of mono-olein, di-olein (9c) and tri-olein (9c) with a purity of 99% were purchased from Larodan Fine Chemicals, Malmö, Sweden. Internal std. 2, a mono-di mixture from mainly C14- (3.9%) C16- (30.0%) and C18- (61.0%) fatty acids, containing 46.4 wt% $MAG_{total,}$ 41.1 wt% DAG_{total} and 11.5 wt% TAG_{total} was kindly provided by Danisco A/S, Brabrand, Denmark. All chemicals used were of analytical grade with a purity of 95-99% obtained from VWR Internal Ltd., Albertslund, Denmark.

Evaluation of the solvent for the TLC-FID separation. Different solvents in various ratios are suggested in the literature for separation of neutral lipid samples, containing MAG, DAG, and TAG (Freedman *et al*., 1984; Nishiba *et al*., 2000; Peyrou *et al*., 1996; Striby *et al*., 1999; Iatroscan, 2001). The most suitable solvent, applicable to single step separation of samples from glycerolysis on S-III chromarods®, was identified by screening of different solvent -combinations and ratios. Some of the most useful chromatograms obtained from this solvent screening are shown in Figure 1.



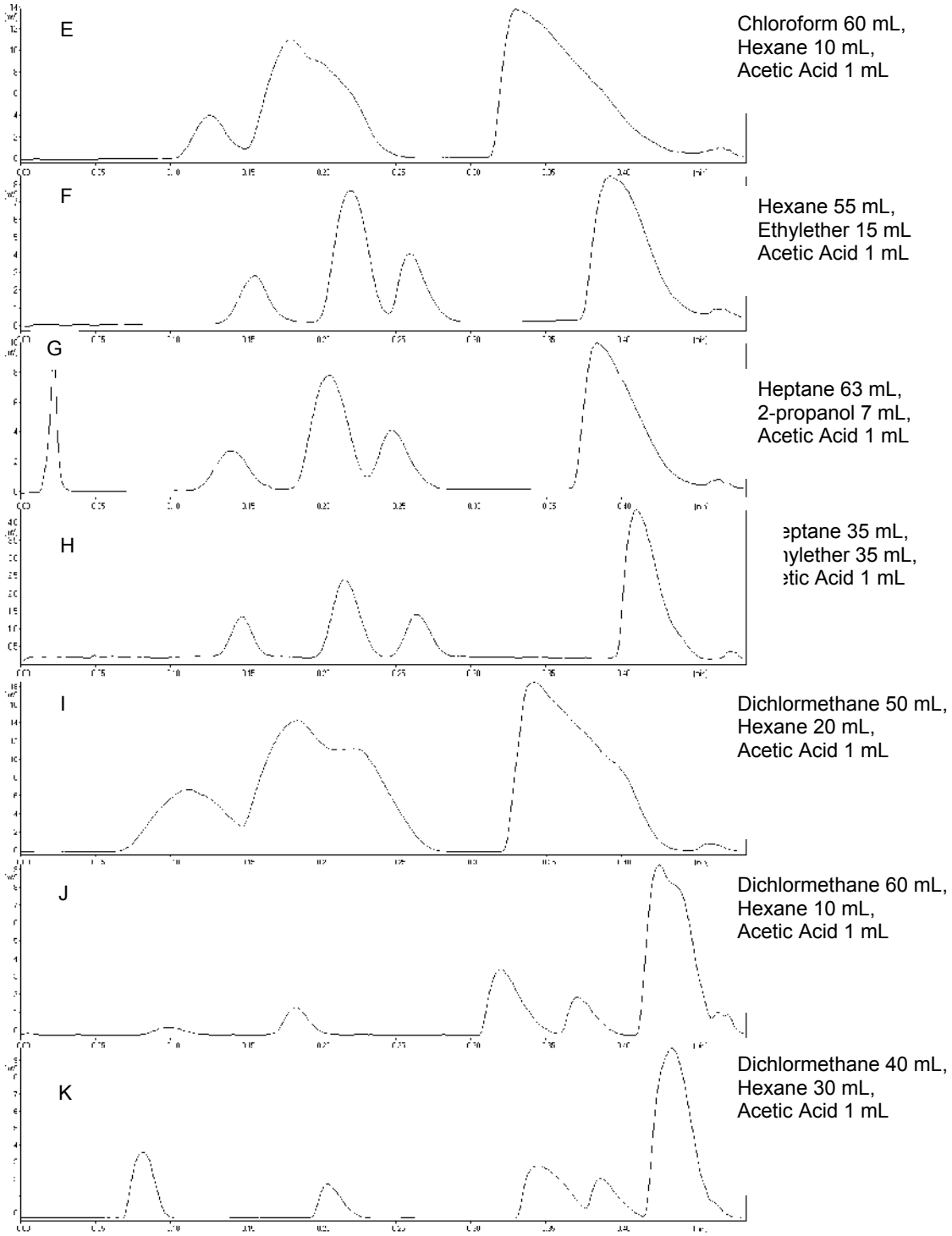


Figure 1: TLC-FID chromatograms obtained from separating MAG, DAG and TAG components (Internal std. 2, Danisco A/S, Denmark) in various developing solvents stated next to the chromatogram.

From the solvent screening hexane 55 mL: ethylether 15 mL: acetic acid 1 mL (Fig. 1F), heptane 63 mL: 2-propanol 7 mL: acetic acid 1 mL (Fig. 1G) and n-heptane 35 mL: diethylether 35 mL: acetic acid 1 mL (Fig. 1H) were considered capable of good peak separation (Figure 1). Two of these solvents (Fig 1F and 1H) included highly flammable and explosive ether, associated with major/extensive hazard precautions. However, further investigations showed a sharper and clearer peak separation in the ether containing solvents compared to the others. Furthermore, all qualified solvent mixtures were related to flammable and harmful risks and it was proved impossible to find non-toxic alternatives. Thus, n-heptane 35 mL: diethylether 35 mL: acetic acid 1 mL was selected as the most suitable solvent based on clear and sharp peak separation ability.

The selected solvent for the TLC-FID separation consisted of equal amounts of the relatively non-polar n-Heptane with an Octanol-Water Partition Coefficients (log P value) of 4.66 and the more polar Diethylether having a log P value of 0.89 (KBA, 2007; Lide, 2007). This implies a 'medium' polarity of the solvent. A more elaborating description of the Log P value can be found in Paper I. The glycerolysis samples were dominated by MAG, DAG and TAG components with very non-polar long chain fatty acids (LCFA) (for instance oleic acid with a log P value of 7.64) (Lide, 2007). Although MAG contains these very non-polar LCFA's the corresponding polar glycerol unit ensure an overall 'medium' polarity of the MAG components. This MAG polarity obvious exceeds the polarity of the DAGs which exceed the polarity of the TAGs. Hence, the selected solvent favors the DAG and especially the MAG migration on the rods from the much less polar TAG- and FFA compounds. From this, the MAG, DAG, TAG and FFA peaks were identified as illustrated in Figure 2.

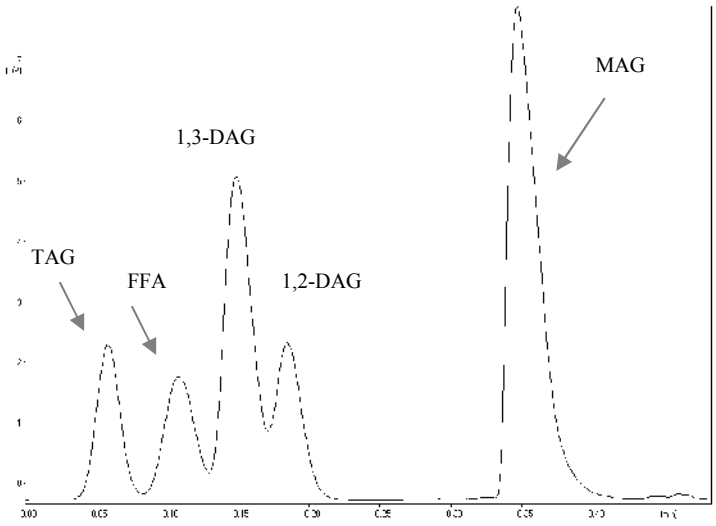


Figure 2: Peak identification of a TLC-FID chromatogram obtained from a mixture of: 10,1 mg internal std 2 from Danisco A/S and 1,1 mg/mL C16 and C18 free fatty acids (FFA).

Trials were made to obtain a sharp separation between the two DAG isomers (1,2-DAG and 1,3-DAG) but turned out unsuccessfully. Hence, the sum of the two DAG isomers (DAG_{total}) was chosen as expression for the DAG amount in the sample. Tests were conducted with glycerol added to the lipid samples to investigate the ability of obtaining a glycerol peak in the chromatogram as well. However, it failed since the very polar glycerol migrated to the very edge of the chromatogram, only partly detectable for the FID. It was attempted to diminish the migration of the glycerol by using less polar solvents. It hereby succeeded to cover glycerol as well as lipids in the same chromatogram. However, it also resulted in overlapped lipids peaks. Hence, it was believed necessary to leave the glycerol identification out of the analysis to maintain satisfactory lipid separation. Multiple separation steps were considered to obtain well separated glycerol and lipid components in the same chromatogram. However, this required prolonged analytical time consumption and hereby eliminated the simplicity and rapidity of the analysis. Based on this, the multiple separation steps were omitted from the method optimization.

Quantification of MAG, DAG and TAG from standard curves. Standard curves were made from pure mono-, di- and triolein solutions to confirm the expected linearity of the TLC-FID method and to quantify the MAG, DAG and TAG compounds. Concentration ranges from 0.1- 200 mg/mL were tested. Concentrations higher than approximately 40 mg/mL lead to very high responses above 60.000 mV outside the suppliers recommended measuring area. Hence, a linear standard curve was made from concentrations from 2 mg/mL to 40 mg/mL standard, giving peak counts from 200-65,000 mV, as illustrated in Figure 3.

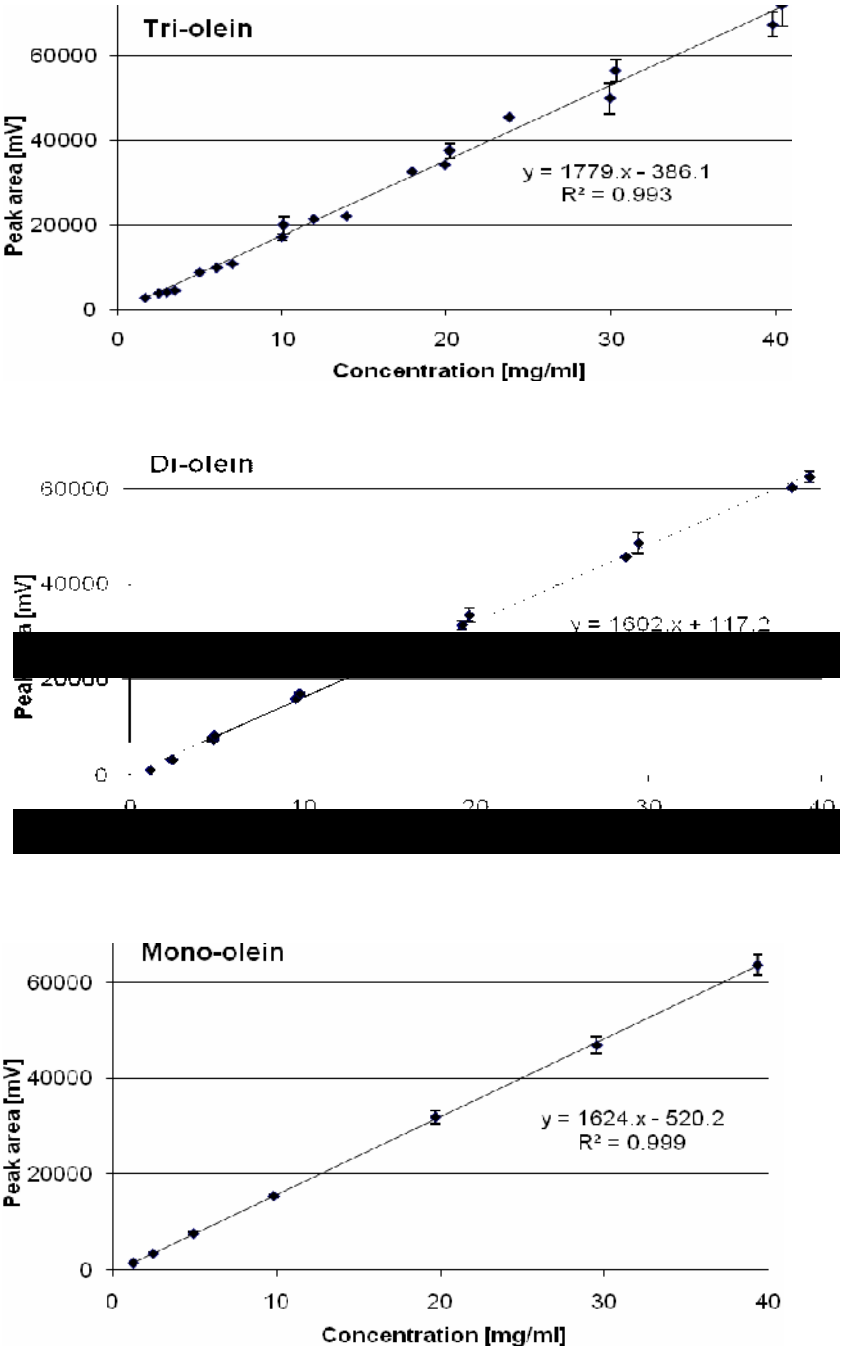


Figure 3: Linear standard curves obtained for tri-, di- and mono-olein in concentration range from 0 mg/mL – 40 mg/mL. Error bars represent STD from double determinations.

The measured peak areas were compared to a calculated value based on the regression lines from the standard curves, shown in Table 1.

Table 1: Example of measured peak areas ± STD from double determinations of 10 mg/mL pure mono-, di- and tri-olein standard compared to calculated values from the regression line.

Compound	Conc mg/mL	Measured Peak area	Calculated peak area
MAG	9.8	16319 ± 649	15137
DAG	9.8	16965 ± 310	15754
TAG	10	17165 ± 718	17712

The pure standards showed good linearity between peak area and sample concentration (Figure 3) and it was possible to quantify the lipid compounds with an uncertainty of maximum 7.2 Area% (Table 1). However, it is worth noticing that the TAG regression line had a higher slope compared to the MAG and DAG regressions lines (Figure 3). This indicates that different responses are obtained depending on the compounds measured.

The three pure standards were mixed in varying concentrations to test if a similar linearity was observed when mixed. At concentrations higher than 20 mg/mL, peaks overlapped, why it was concluded that the compound concentration should not exceed 20-25 mg/mL. The achieved standard curves from concentrations below 20 mg/mL are shown in Figure 4.

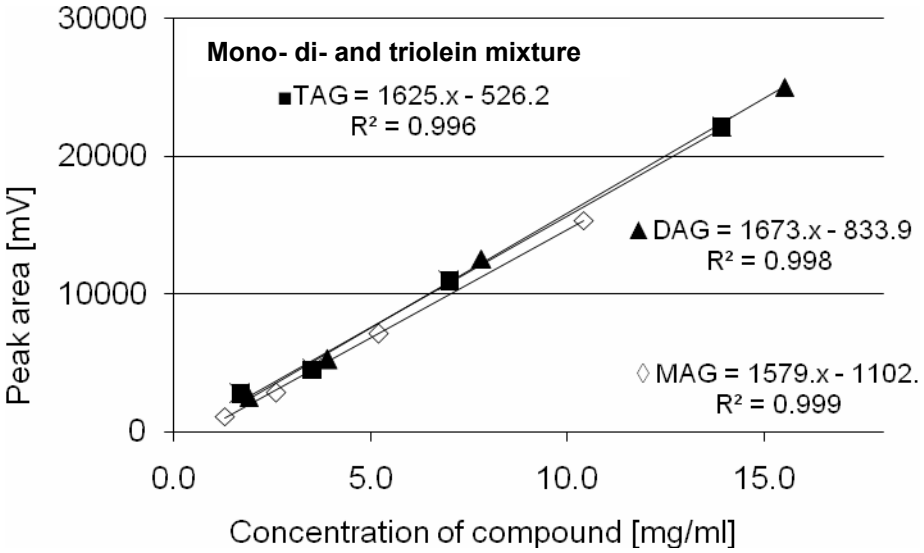


Figure 4: Linear standard curves obtained from a mixture of pure tri-, di- and mono-olein (13.9, 15.5 and 10.4 mg/mL). Results are based on double determinations.

Relative good linearity was observed for the three standards in a mixture as well. However, it was only possible to quantify the lipid compounds with a very high uncertainty degree up to 15% when measured and calculated peak area (from regression line) was compared. Also, a shift in the slope order was observed when the regression lines from the mixture were compared to the ones from separate compounds. In the mixture, the slope from the DAG regression line was highest while the MAG regression line showed the lowest slope (Figure 3). This is in contrast to the pure standards where the TAG regression line had the highest slope and the DAG regression line had the lowest slope (Figure 4). This indicates that the response varies not only according to compound measured but also depending on the concentration of each component when mixed. Based on these findings, quantification of the compounds from the standard curves were believed associated with too many errors and thus found inappropriate.

Quantification of MAG, DAG and TAG from propylgallate as internal standard. The internal standard propylgallate (PRO) was tested as an alternative to standard curves for compound quantification. A chromatogram obtained from addition of propylgallate as internal standard is illustrated in Figure 5.

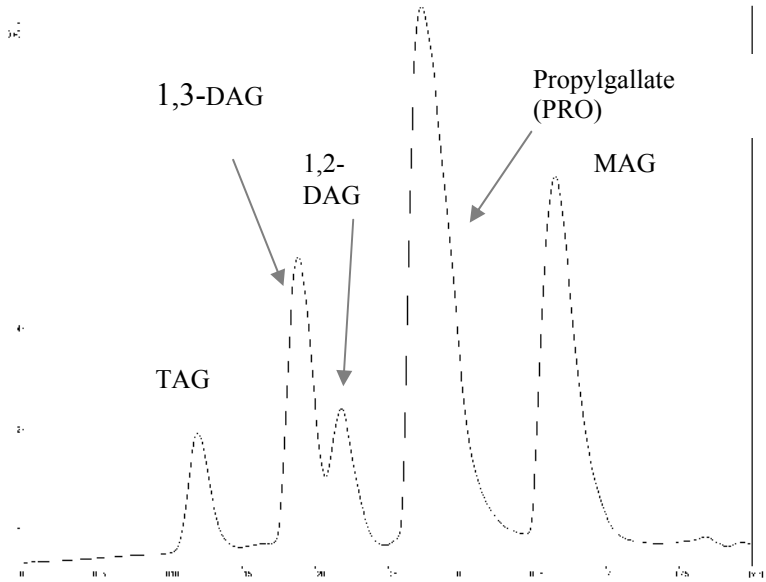


Figure 5: TLC-FID chromatogram obtained from a mixture of 9.34 mg/mL propylgallate and 10.1 mg/mL internal standard 2..

Propylgallate showed very good separation from the lipid compounds which made it well suitable for Internal Standard (IS) applications in the actual samples (Figure 5). To identify a response factor ($R_f = A_c \cdot C_{is} / A_{is} \cdot C_c$) required for the quantification, the propylgallate concentration measured (C_{is}) and the measured peak area (A_{is}) were compared to similar values for MAG, DAG and TAG (C_c and A_c), summarized in Table 2.

Table 2: Measured peak areas and different calculated values to identify a response factor defined as $R_f \cdot C_c / C_{is} = A_c / A_{is}$ from a mixture of 9.34 mg/mL propylgallate (PRO) and 10.1 mg/mL internal standard 2, Danisco A/S based on double determinations.

Compound	Conc. mg/mL	Peak area	C_c / C_{is}*	A_c / A_{is}*	R_f
TAG	1.1615	1210 ± 25	0.124	0.114	0.919
DAG	4.1511	5194 ± 111	0.444	0.490	1.104
MAG	4.6864	6082 ± 195	0.502	0.574	1.143
PRO	9.34	10590 ± 252	1	1	1

**C= Concentration, c= compound (MAG, DAG or TAG), is= internal standard (PRO), A= Peak area*

The obtained response factors varied from compound measured with MAG and DAG having factors very close to each other of 1.1 while the TAG response factor was lower, close to 0.9 (Table 2). This indicates that TAG generates a higher response than MAG and DAG, at similar concentrations. To test if these response factors could be commonly used at other concentration levels, similar calculations were made in a broader concentration range. The results from the calculations are illustrated in Figure 6.

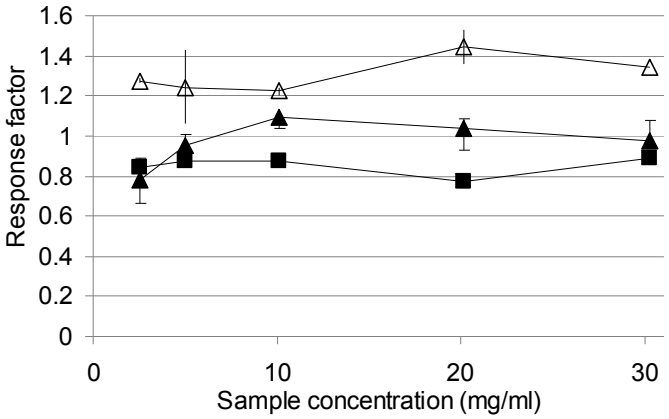


Figure 6: Calculated response factor for MAG, DAG and TAG from different sample concentrations of internal standard 2,Danisco A/S and propylgallate (ratio 1.1 mg: 1 mg). Error bars represent STD from double determinations. ▲= MAG, Δ=DAG, ■=TAG.

Unfortunately, the achieved response factors varied depending on the sample concentration (Figure 6). Hence, it failed to identify precise response factors independent of the sample concentration. Thus, it was concluded too ambiguous to rely on a response factor based on the propylgallate concentration for MAG, DAG and TAG quantification.

Determination of the MAG, DAG and TAG distribution from TLC peak areas. The uncertain compound quantification made an estimation of the MAG, DAG and TAG distribution by a non-quantitative approach desirable. Hence, the relationship between measured peak areas from MAG, DAG and TAG was investigated in more details. A mixture of MAG, DAG and TAG (internal std. 2, Danisco A/S) in different concentrations and with a distribution likely to the one after a glycerolysis reaction was analyzed for the relative lipid distribution based on peak areas.

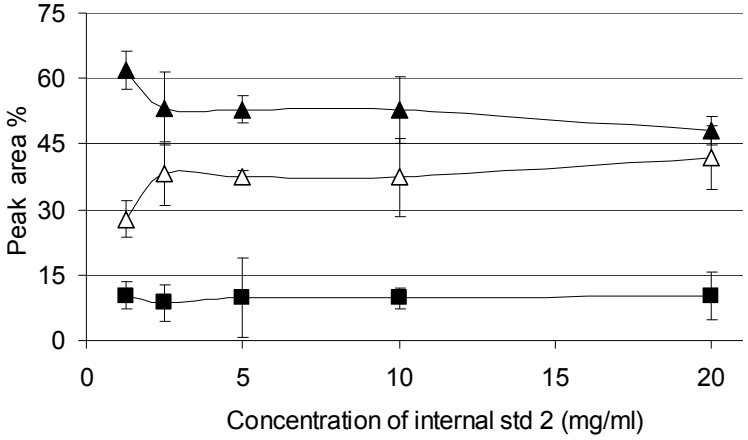


Figure 7: Measured relative distribution of lipid components from internal std 2, Danisco A/S, containing 46.4 wt% MAG, 41.1 wt% DAG and 11.5 wt% TAG, in varied concentrations from 1.2-20 mg/mL. Error bars represent STD of double determinations. ▲= MAG, Δ=DAG, ■=TAG.

The concentration of the MAG-, DAG- and TAG-mixture strongly affected the measured relative compound distribution (Figure 7), confirming a non-linear relationsship between weight- and area%. In general, TAG was found sligthly underestimated (from 8.7-10.3 area%) while MAG in general was overestimated (48.1-61.9 area%) compared to the actual values of 11.5wt% and 46.4wt%, respectively (Figure 7). This indicates that smaller sample amounts in general yields smaller responses than larger sample amount. The best fit of the lipid distribution was obtained at a sample concentration of 20 mg/mL (Figure 7). Here the measured distribution of 48.1 area% MAG, 41. 8 area% DAG and 10.1 area% TAG was almost equal to the actual distribution of 46.4 wt% MAG, 41.1 wt% DAG and 11.5 wt% TAG. In the concentration range from 10-20 mg/mL the peak area counted approx 1,000-15,000 mV in response on the TLC. Apparently, this gave a relative good estimation of the compound distribution, allthough variation up to 10-15% in peak area was observed. From this it was concluded

that measurement of the peak areas was useful for a quick and roughly estimatation of the relative MAG, DAG and TAG weight-% distribution.

Accuracy of the TLC-FID technique. To identify the accuracy of the TLC measurement four repeated measurements were made of each sample in varies concetnrations. The obtained results are shown in Table 3.

Table 3: Measured average peak areas ± STD and calculated coefficient of variation (CV) from varied concentrations of internal standard 2, Danisco A/S.

Conc.	TAG		DAG		MAG	
(mg/mL)	AVERAGE	CV*	AVERAGE	CV*	AVERAGE	CV*
1.3	125 ± 4	3.0	462 ± 43	9.4	719 ± 34	4.8
2.5	250 ± 13	5.4	925 ± 59	6.3	1464 ± 105	7.2
5.0	549 ± 14	2.5	2084 ± 17	0.8	2970 ± 57	1.9
10.0	1257 ± 78	6.2	4861 ± 217	4.5	6876 ± 210	3.1
20.0	2839 ± 106	3.7	12045 ± 597	5.0	14194 ± 452	3.2
29.9	4143 ± 257	6.2	17429 ±1567	9.0	19956 ± 1519	7.6
40.0	6351 ± 455	7.2	26707 ± 1753	6.6	29869 ± 1852	6.2

**Calculated as STD*100/Average value based on four determinations.*

The repeated measurements showed an coefficeient of variation up to 9.4%, indicating a relative high uncertainty level of the analytical method (Table 3). Although a semi-spotter was used to simplify the sample application, the very low sample amount of just 1 µl easily lead to errors. Physical contact between the syringe pin and the rod deemed of great importance for fully sample transfer, illustrated in Figure 8.

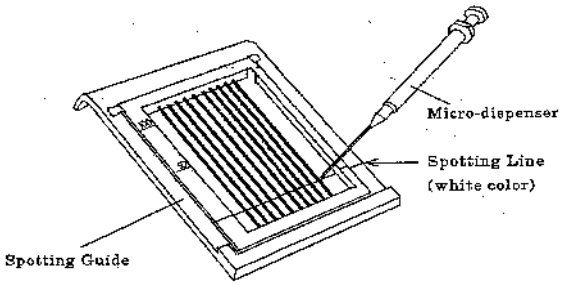


Figure 8: Illustration of the spotting procedure for the TLC-FID analysis.

In addition, a relatively fast application ('fast speed') was required during the spotting procedure to avoid spontanous evaporation of the sample diluted in the volatile chloroform-methanol mixture.

Evaluation of the TLC-FID method compared to the GC-FID. Arisen from the lack of glycerol identification and uncertain compound quantification using TLC-FID, GC-FID was implicated over time as a more detailed and quantitative analytical technique. A chromatogram obtained from the GC-FID analytical procedure is illustrated in Figure 9.

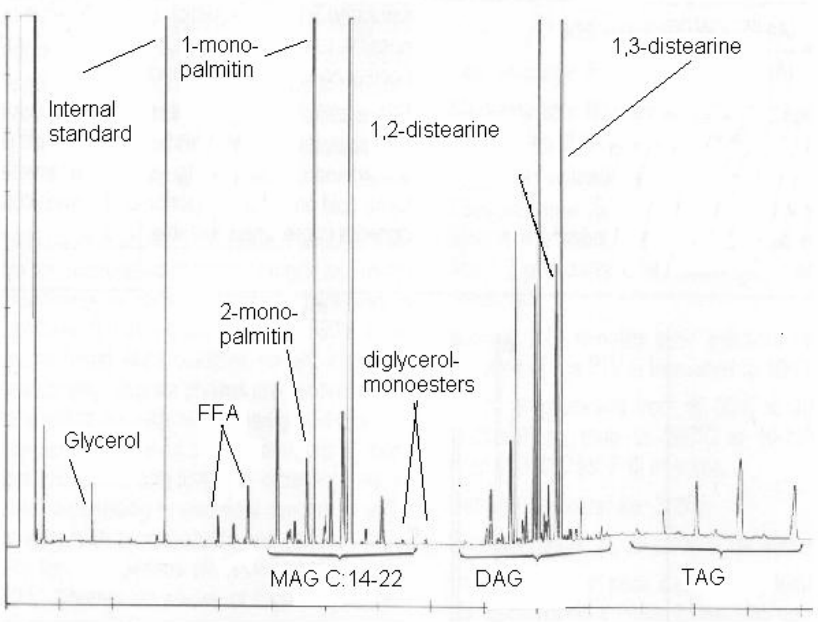


Figure 9: Illustration of a chromatogram obtained from analysis of a sample after a glycerolysis reaction by GC-FID.

Among the obvious benefits of the GC-FID techniques compared to TLC-FID is the possibility to measure different isomer (1-(3)-MAG versus 2-MAG), identify the chain length of the fatty acid and quantifies the glycerol content, all in the same run (Figure 9). In the actual investigated process, the glycerol conversion is of great importance for the evaluation of the reaction since glycerol represents one of the major reactants and is a plausible by-product after reaction. Hence, lack of glycerol content can be fatal for the conclusions made from a screening of for example different molar ratios of glycerol to oil, as illustrated in Figure 10.

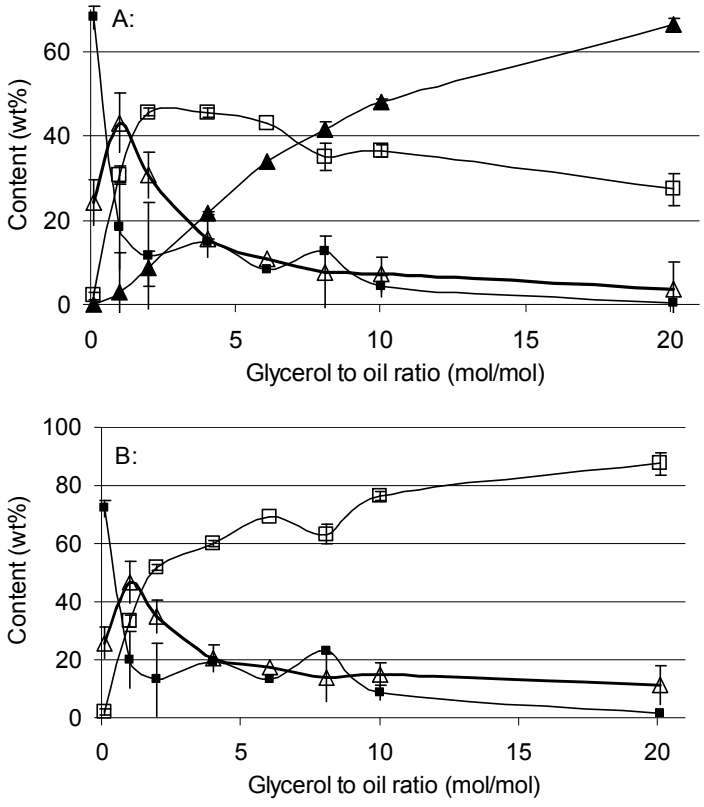


Figure 10: Compound distribution after glycerolysis in a tert-butanol: tert pentanol 80:20 vol% batch reaction system at different substrate ratio (reaction time 180 min) from GC-FID measurements. A: Normalization of the measured MAG, DAG, TAG and Glycerol content (total set to 100 wt%). B: Similar to A, but normalized without the glycerol content. ■ *= TAG,* Δ*=DAG,* □*=MAG,* ▲*=Gly.*

Figure 10A demonstrates that a glycerol to oil molar ratio higher than 5 decreases the MAG content from a glycerolysis reaction, due to an increase of retained (non-reacted) glycerol. Figure 10B exhibit an increase in the MAG content with increasing glycerol to oil ratio, contrary to Figure 10A. Hence, exactly same results evaluated with the glycerol content omitted indicate conflicting findings. By using the TLC-FID analytical techniques it can be concluded that very high glycerol to oil molar ratios are solely beneficial for the obtained MAG content. Thus, it is very important to be cautious when TLC-FID is used, and to consider the consequences to refrain from glycerol evaluation. Also, these findings should be kept in mind when the literature is evaluated since it is commonly used to analyze the acylglycerols from glycerolysis without the glycerol content included.

In the actual process, where the glycerol content is of main concern, the GC-FID indeed is a well suited method, providing a very precise and detailed picture of the entire compound composition. However, equipment limitations and the need for rapid results make TLC-FID suitable for quick preliminary answers about the acylglycerols composition. To verify the applicability of TLC-FID for MAG, DAG and TAG estimation based on peak areas, identical samples from a glycerolysis reaction were analyzed by TLC-FID and GC-FID. Results are shown in Figure 11.

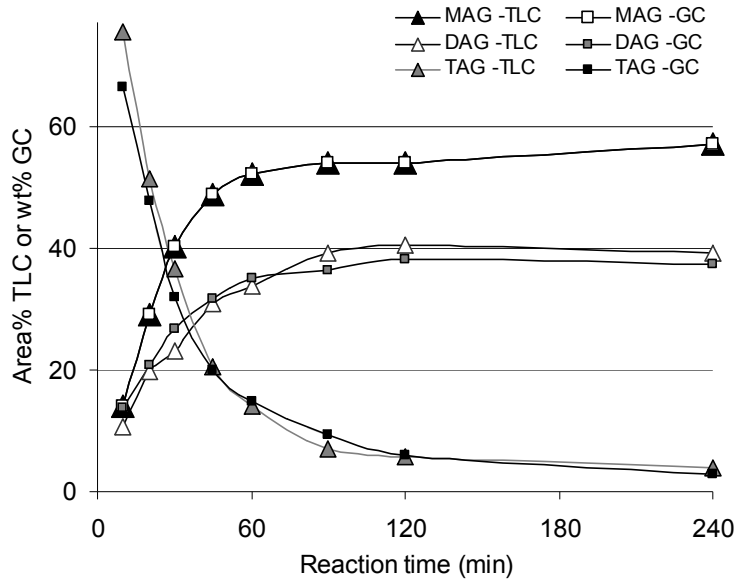


Figure 11: Comparison of TLC measurement of MAG, DAG and TAG with GC measurement on identical samples from glycerolysis in a tert-butanol batch system with a glycerol to oil molar ratio of 2. Sum of MAG , DAG and TAG are normalized and set to 100% with wt% for GC and area% for TLC.

As seen from Figure 11, the normalized peak area of MAG, DAG and TAG from TLC fitted very well to the normalized wt% of MAG, DAG and TAG from GC. Hence, it was verified that TLC serve well as a simple and fast method to estimate the relative lipid distribution from glycerolysis.

Conclusion

The most suitable developing solvent for the TLC-FID analysis of samples from glycerolysis was found to be a n-Heptane 35 mL: Diethylether 35 mL: Acetic acid 1 mL mixture. It was proven infeasible to substitute this developing solvent with a non-toxic alternative and still maintain a clear and sharp peak separation of the acylglycerols. By using the TLC-FID, it failed to quantize the MAG, DAG and TAG compounds from standard curves of pure mono-di and triolein as well as the internal standard propylgallate. Only semi-quantitative results associated with high degree of errors were achieved on the TLC-FID and thus found inappropriate. However, a non-quantitative approach of the TLC-FID method was found suitable for a quick screening method to roughly estimate the relative lipid distribution of MAG, DAG and TAG based on a normalization of the measured peak areas. Nevertheless, a high uncertain degree of the TLC-FID measurements with a inaccuracy up to approximate 10% must be accepted.

In the actual process, glycerol represents one of the main reactants making the GC-FID method well suited to obtain a detailed picture of all components included. GC-FID has the advantages compared to TLC-FID of being capable of quantify glycerol and all the acylglycerols in one run. All things considered, TLC-FID is believed suitable for rapid detection of the lipid distribution while GC-FID is recommended when more details is required.

Acknowledgement

Bodil Alrø, Danisco A/S, Brabrand, Denmark is gratefully acknowledged for her outstanding technical assistance and support with the GC-FID analysis and valuable advice in analytical matters. Anni Jensen, BioCentrum-DTU is thanked for technical assistance with the TLC-FID equipment and useful support in general.

Literature

Freedman, B;. Pryde, E. H and Kwolek. W. F. (**1984**). Thin-Layer Chromatography Flame Ionization Analysis of Transesterified Vegetable-Oils. *J. Am. Oil Chem. Soc.* 61 (7),1215-1220.

Iatroscan **(2001)** Instrument Application Appendix-II No. 5: Analysis of Lipids by the Iatroscan. In *Instruction Manual for Iatroscan MK-6 and Iatroscan TLC/FID.*

KBA, 2007 http://www.kemibrug.dk/KBA/CAS/60-29-7. Accessed 07.12.2007

Lide, D.R. (**2007**). Octanol-Water Partition Coefficients. In *Handbook of Chemistry and Physics.* Ed. Lide D.R, CRC Press, 87th ed. [on-line] section 16.

Nishiba, Y.; Sato, T.; Suda, I. (**2000**) Convenient Method to Determine Free Fatty Acid of rice using Thin-layer Chromatography and Flame-ionization Detection System. *Cereal Chem.* 77 (2), 223-229.

Peyrou, G., Rakotondrazafy, V., Moulonoungui, Z. (**1996**). Separation and Quantitation of Mono-Di- and Triglycerides and Free Oleic Acid using Thin-layer Chromatography with Flame-Ionization Detection. *Lipids*, 31, 27-32.

Striby, L.; Lafont, R.; Goutx, M. (**1999**). Improvement in the Iatroscan Thin-layer Chromatographic-Flame Ionisation Detection Analysis of Marine Lipids. Separation and Quantitation of Monoacylglycerols and Diacylglycerols in Standards and Natural Samples. *J. Chromatogr. A*. 849 (2), 371-380.

Description of the TLC-FID method

Fields of application:

Thin-layer chromatographic (TLC) -flame ion detection (FID) is a simple and convenient method for separation and quantification of neutral lipids like triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG) and free fatty acids (FFA) from fats and oils. The here described method is optimized to analyze different lipid standards and various samples from the glycerolysis reaction of glycerol and vegetable oils, rich in unsaturated long chain fatty acids.

Principle:

The TLC - FID analyser combines the separation capabilities of conventional TLC with the quantification power of the flame ion detector (FID). The overall principle is illustrated in In Fig. 1.

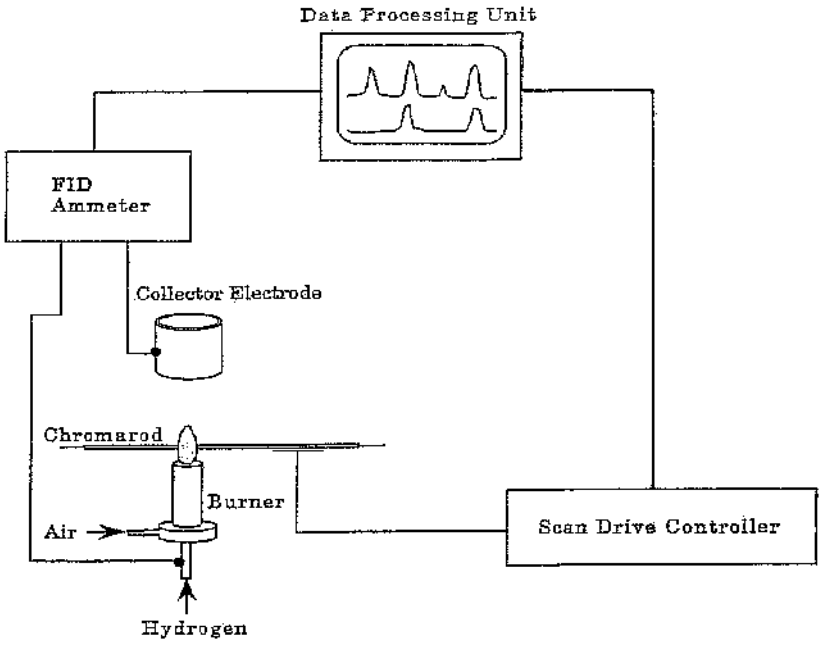


Figure 1: Illustration of TLC-FID principle.

Samples are spotted onto thin layer rods (Chromarods) (stationary phase) and are then separated on the rods by a solvent (mobile phase). The rod bearing separated sample traverses the hydrogen flame to ionize the sample by the energy of a hydrogen flame. Owing to high voltage on FID electrodes, minus-ions (-) moves to the burner and plus-ions (+) to the collector electrode, which generates minute ionic current on the electrodes in between. The ions current flow between the burner and collector are proportional to the mass of the component/substance introduced in hydrogen flame (reduced C-atoms) and are converted to mV by current voltage amplifier. Output is send to a data processing unit.

Apparatus:

The Iatroscan MK6 apparatus and the ancillary equipment, all supplied by SES GmbH, Bechenheim, Germany, are partly illustrated in Fig 2 and includes:

Iatroscan MK-6 from Iatron Laboratories, Tokyo, Japan

SD-5 Rod Holder with 10 ChromarodSIII® rods made of quartz glass coated with a 75 μm thin layer of soft glass powder mixed with an adsorbent silica gel. Mean diameter of the micro pores are 60 Å and an average particle size 5 μm.

Semi automatic sample spotter model SES 3202/IS-02 including a spotting guide and micro-dispenser

TK-8 Chromarod dryer

DT-150 Development tank lined with a filterpaper

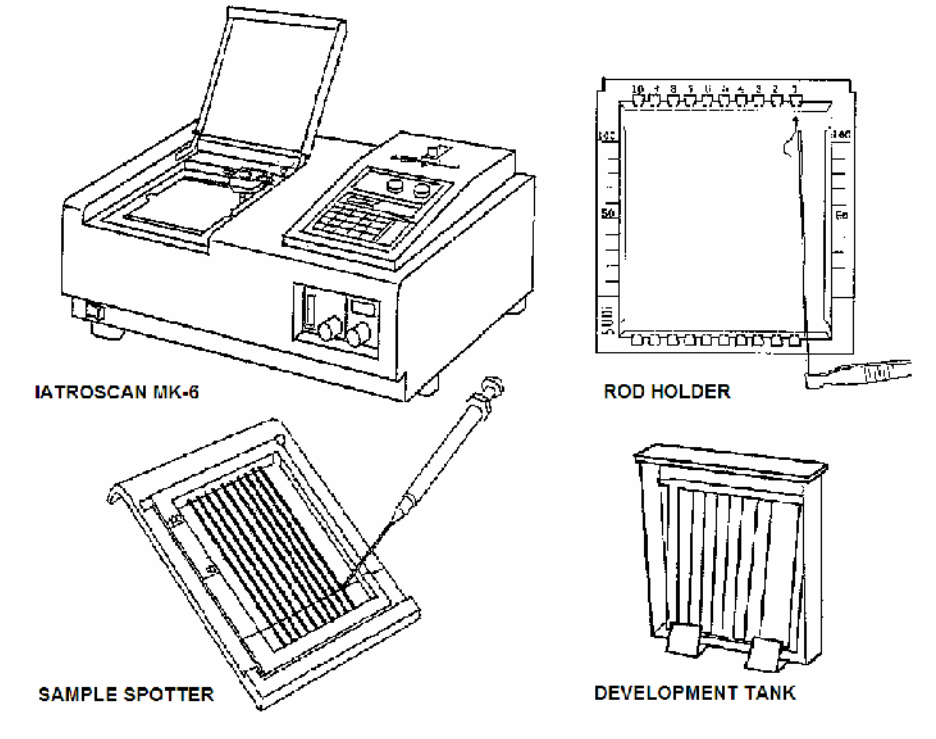


Figure 2: Illustration of the equipment used for the TLC-FID analysis

Reagents:

Solvent for sample dilution:
Chloroform: methanol 85: 15 vol%

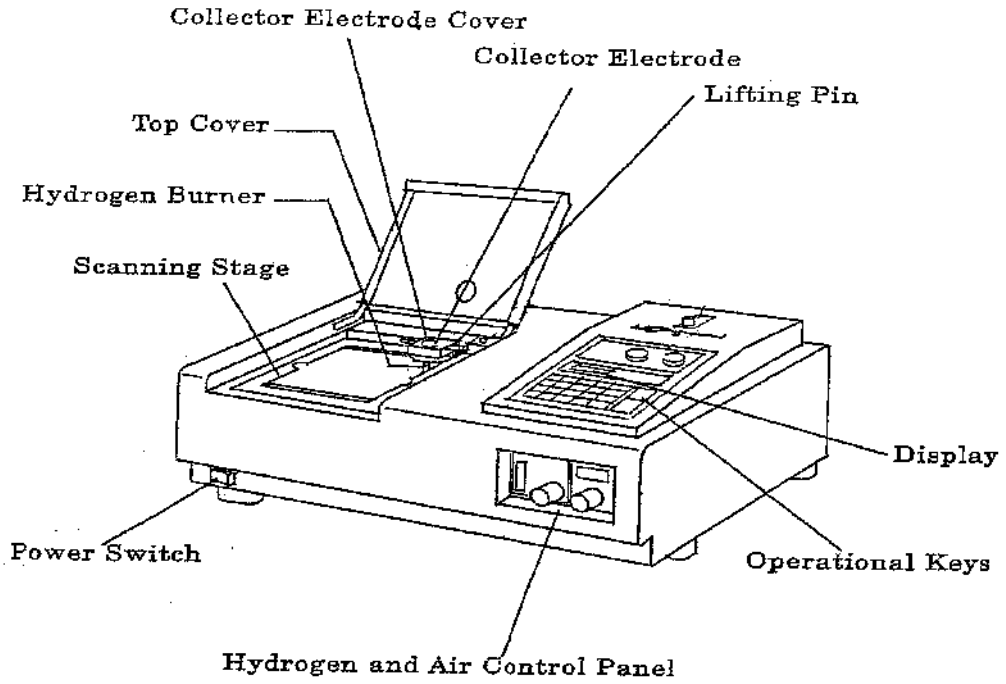
Solvent for development tank:
Diethyl ether: n-Heptane: Acetic acid 35:35:1 vol%

Overall procedures:

20 mg sample (flushed with nitrogen to remove eventually solvent from reaction) were diluted in 1 mL chloroform:methanol 85:15 (v/v). 1 µL diluted samples were transferred to the ChromarodSIII® quartz rods by the semiautomatic sample spotter. Samples were separated on the rods for 25 minutes by approximately 70 mL solvent stored in a DT-150 development tank, covered with filter paper. The solvent consist of *n*-heptane: diethyl ether: acetic acid 35:35:1 v/v. The rods were dried for 5 min at 120°C in a TK-8 chromarod dryer before traverses the hydrogen flame on the Iatroscan MK-6. The ionized sample components were detected in a flame ionization detector (FID) and converted to mV by a voltage amplifier. Subsequently, output was send to a PC equipped with the SES I-Chromstar® 6.0 Software. Results were expressed as area percentages of double determinations and calculated from normalization of the MAG, DAG and TAG area (= total set to 100%).

More detailed operating procedures can be seen in the following user manuals (In Danish as well as English).

Betjeningsvejledning til Iatroscan MK-6 (TLC-FID)



1. Prøveforberedelse:

1.1. Afdamp eventuel tilstedværende solvent vha. gennemblæsning med nitrogen.
1.2. Forvarm evt. Proven I vandbad eller mikrobølgeovn under omrøring til max. 70°C.
1.3. Omrør proven omhyggeligt til den er homogen.
1.4. Afvej 20 mg olie prove og opløs i 1 ml opløsnings solvent
1.5. Bland grundigt evt på whirl mixer.

2. Klargøring af solvent til prøveseparation:

2.1. Bland ca. 70 ml solvent.
2.2. Hæld solvent i separationsbeholderen og dæk hurtigt åbningen med et låg.
2.3. Beklæd en af beholderens sider med et filter papir ca. 14 x 17 cm
2.4. Las solventet fugtiggør filterpapiret helt for at mætte beholderen og sørg for at beholderen er lukket med et låg.

3. Opstart:

3.1. Åben for hydrogen tilførslen via hanen på væggen
3.2. Tænd for Iatroscan på POWER 1-knappen, med "electrode collector" i sænket position.
3.3. Juster AIR CONTROL til 2.0 L/min.
3.4. Juster HYDROGEN CONTROL til 160 mL/min.
3.5. Juster SCAN SPEED til 30 s/rod via betjeningspanelet (tast 2=30 sec).
3.6. Åben "top cover" på Iatroscan og løft "collector electrode" ved hjælp af løftepind (Lifting pin)
3.7. Tænd for hydrogen brænder med lighter.
3.8. Tænd for ROD DRYER TK-8 (varmeskab) på 120°C

4. Opsætning i-Chrom-Star 6.0 program på computer

4.1. Tænd for Computer enhed og åben i-Chrom-Star program.
User-ID = "enter-knappen"
4.2. Klik Edit files:
Første gang oprettes en folder i dit navn/initialer eller lign.
MET: Run table: GC, prove ID mm udfyldes.

Hver kørsel (max 10 stave) gemmes (Save as) alle i samme navn.

INT: Slice width: 50, delay time: 0, run time: 0,55, Gemmes screen scale: 6.
CAL. Base: Area, Method: Percent, Univ. resp. fact:1,
Min. area: 100, Conc. 1 Percent window: 5%, min time: 0.1

5. Blank scanning

5.1. Hæv "collector electrode" vha af løftepind.
5.2. Placer "stav-holderen" i scanning position ved at starte med at placere den nedre del af stav-holderen og derefter den øvre del.
5.3. Sænk "Collector electrode" til original position.
5.4. Indstil Iatroscan til BLANK SCAN via betjeningspanel og indstil ønskede antal scanninger pr. stav (1-2 gange pr. stav) via TAL-tasterne + ENTRY knappen.
5.5. AUX SGN slås fra for at undgå registrering på computerskærm og herefter trykkes på START.

6. Prøve påsætning

6.1. Placer stav-holder i SAMPLE SPOTTER.
6.2. Tilfør vha af sprøjten 1 µL prøveopløsning ved 0-mærket på rammen af stav holder.
6.3. For at minimere påførsels usikkerhed er det MEGET vigtigt at prøven tilføres med "hurtigste=normal" hastighed, og at der er direkte kontakt mellem nål og Chromarod stav.
6.4. Forud for hver prøvepåsætning skal nålen renses med opløsningsmiddel, der er passende i relation til pågældende prøve eksemplevis chloroform.

7. Prøve separation i beholder med solvent

7.1. Mix 70 mL eluent og tilfør det til fremkalderbeholder.
7.2. Dæk den ene side med et filterpapir og lad blandingen stå til filterpapiret er helt gennemfugtet (ligevægtsbetingelse).
7.3. Placer stav-holderen i beholderen i oprejst position.
7.4. Påsæt hurtigst muligt låget på beholderen.
7.5. Fjern stav-holderen når prøverne har vandret til nær 100-mærket på stav-rammen. NB: Prøverne MÅ IKKE overstige 100-mærket.
7.6. Overskydende eluent fra stavene fjernes ved opbevaring i 120°C varmeskab i 5 min.

8. Scanning/måling

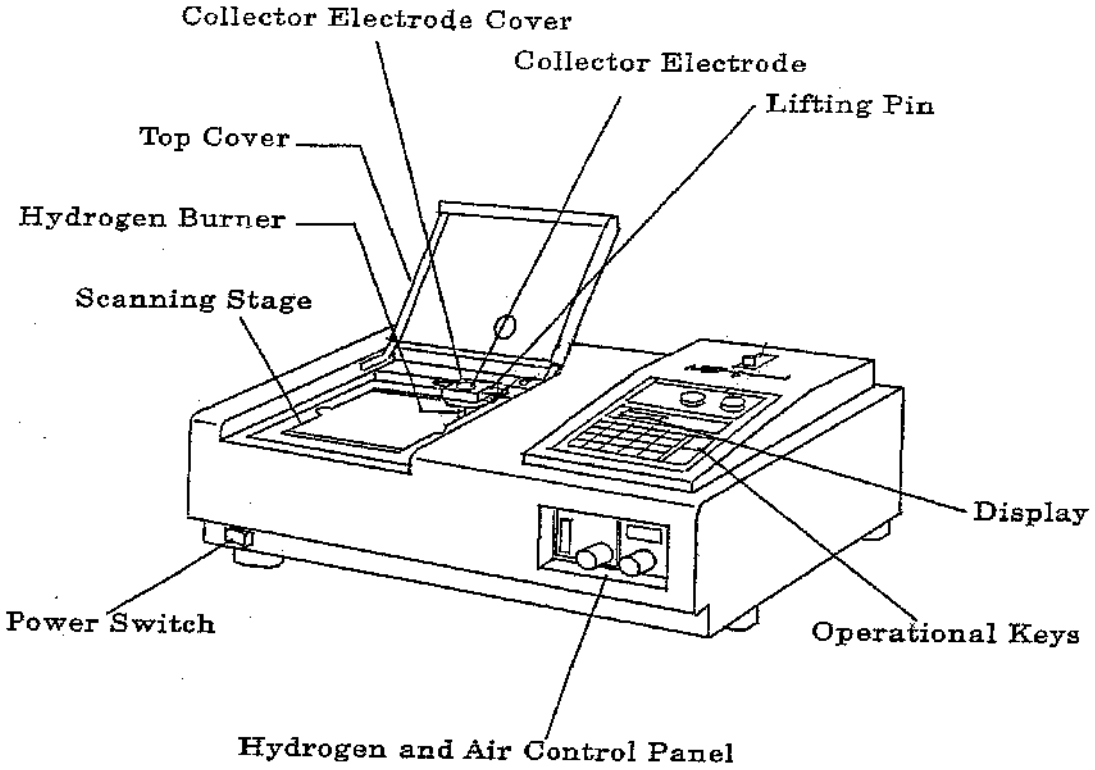
8.1. Hæv "collector electrode" vha. af løftepind.
8.2. Placer stav-holder i "scannings position"
8.3. Sænk "Collector electrode" til original position
8.4. Luk låget
8.5. Juster basislinjen til 0 vha AUTO ZERO tasten på betjeningspanelet
8.6. Forbind Iatroscan med computerskærm vha AUX SGN key
8.7. Tryk på NORMAL SCAN tast.
8.8. Vælg SCAN SPEED til 30 sec/scan og antallet af stave der skal måles vha ROD NO. + TAL-taster + ENTRY
8.9. Tryk på START tast for at måle

9. Afslutning

9.1. Luk for computerenheden
9.2. Luk for hydrogen tilførslen på væggen og tjek at trykket falder til 0
9.3. Sluk på POWER O knappen på Iatroscan

10. Opbevaring af stave

10.1. Opbevar stavene i et lukket kammer hvor de ikke udsættes for støv og lign



1. Preparation of sample:

1.1. Remove solvent from the sample by evaporation by nitrogen flow.
1.2. If needed preheat the sample in waterbath/microwave while stirring to max. 70°C.
1.3. Mix the sample properly to make sure it's homogenous.
1.4. Weight out 20 mg oil sample and dissolve in 1 ml dilution solvent.
1.5. Mix the solution properly for instance on whirl mixer.

2. Preparation of solvent for the separation step

2.1. Mix approx 70 ml solvent for the separation step.
2.2. Pour in the solvent to the development tank and cover the opening with a glass lid immediately.
2.3. Line one side of the TLC development tank with filter paper approx. 14 x 17 cm
2.4. Wet the filterpaper completely to saturate the tank make sure to cover with lid.

3. Initial instrument start up:

3.1. Fully open the main valve on the hydrogen gas cylinder
3.2. Turn the Iatroscan power switch to ON, with the collector electrode in lowered order.
3.3. Adjust the air flow at 2.0 L/min.
3.4. Adjust the hydrogen flow rate at 160 mL/min.
3.5. Adjust scanning speed to 30 s/rod.
3.6. Open the top cover of the Iatroscan and lift up the collector electrode by the lifting pins.
3.7. Ignite the hydrogen burner with a long-grasp lighter. Watch out for burn.
3.8. Turn ON the Iatrocorder (data processing unit)

4. Set up of recording conditions

4.1. Edit files-set met: Run table: GC, and fill out sample table.
4.2. set int: slice width: 50, delay time: 0, run time: 0,55, screen scale: 6.

5. Blank scanning

5.1. Make one blank scan before each run in order to remove organic substances and "activate" the Chromarods
5.2. Place the Rod holder in the scanning stage by raising the collector electrode by the insulated pin. Then fit the bottom part of the holder to lower slot and afterwards the top of the holder. Lower the collector electrode to original position.
5.3. Set the Iatroscan for BLANK SCAN and enter required number – one to two times
5.4. Press AUX SGN in order to avoid processing of data and then START.

6. Sample spotting

6.1. Place the rod holder onto the spotting guide.
6.2. Eject 1 μL diluted sample by a micro-dispenser on the origin points of Chromarods with the white spotting line of spotting guide as guideline.
6.3. To minimize spotting error eject the sample "fast" by the sample spotter and make sure there are surely contact between the micro-dispenser and the Chromarod.
6.4. Prior to every sample spotting the syringe should be washed with chloroform 1-2 times.

7. Sample separation in solvent containing tank

7.1. Place the Rod Holder in the development tank in the upright position. Cover the opening with the lid immediately.
7.2. When the solvent front reaches a height close to the 100-graduation mark on the rod Holder the development is finish and the Rod Holder should be removed from the tank (approx. 25 min).
7.3. Remaining solvent is removed by replacement of the rod holder in a heated oven 120°C for 5 minutes.

8. Scanning

8.1. Raise the collector electrode by the lifting pins
8.2. Place the Rod Holder in the scanning stage
8.3. Lower the collector electrode to the original position
8.4. Close the lid of the Iatroscan
8.5. Adjust the electrical zero point of FID by press AUTO ZERO key on control panel
8.6. Synchronism Iatroscan with data processing unit by press the AUX SGN key
8.7. Set the scan mode by pressing NORMAL SCAN key.
8.8. CAN SPEED to 30 sec/scan and number of rods to be measured
8.9. Press START key for measurement

9. Shutting down

9.1. Turn the main valve on the hydrogen gas cylinder clockwise to shut off all gas pressure. Check the pressure drops to zero and the FID burner is completely extinguished.
9.2. Turn off hydrogen regulator valve on the cylinder (Counterclockwise) and the hydrogen inlet valvet on the rear panel of the Iatroscan.
9.3. Turn off the power switch on the Iatrocorder/data processing unit.
9.4. Turn off the Iatroscan

10. Storage of Chromarods

10.1. 10.1 To prevent dust and organic contamination the Chromarods should be kept in desiccators (without desiccant in it) or a storage chamber.

Appendix II

Reaction kinetics of the enzymatic glycerolysis of vegetable oils and glycerol and sn-specificity

Objective:

To experimentally investigate the reaction kinetics of the enzymatic glycerolysis process by studies of the individual products and reactants in a few selected reactions, initially and at equilibrium conditions.

To clarify the specificity of the Novozym®435 in the present glycerolysis system.

Materials:

Pure MAG, DAG and TAG raw material produced from unhardened Rapeseed oil were kindly provided by Danisco A/S, Brabrand, Denmark. Sunflower oil was purchased from Aarhus United, Aarhus, Denmark. The purity of the MAG, DAG and TAG raw material were 98.6%, 95.8 % and 99%, respectively. The sunflower oil contained 97.1% TAG, 2.5% DAG and 0.4% MAG. The main fatty acid composition of the different raw materials are illustrated in Table 1.

Table 1: Fatty acid composition of sunflower oil and pure TAG, DAG and MAG material.

Fatty acid	TAG rapeseed	DAG rapeseed	MAG rapeseed	Sunflower oil
C15≤	0.3	0.2	0.3	0.1
C16	4.9	4.6	5.1	6.7
C16:1	0.3	0.3	0.3	0.2
C17	0.2	0.3	0.2	0.1
C18	1.8	1.7	1.7	3.7
C18:1	60.3	59.1	59.7	26.3
C18:2	20.7	20.7	20.5	61.2
C18:3	8.7	10.1	9.3	0.4
C20 ≥	2.8	3.0	2.9	1.3
Sum	100	100	100	100

Analytical grade glycerol with purity of minimum 99% was purchased from VWR Internal Ltd., Albertslund, Denmark. *Tert*-pentanol (2-methyl-2-butanol) abbreviated TP with a purity of 96% or 98% and *tert*-butanol (2-methyl-2-propanol) abbreviated TB with a purity of 99% were provided by Sigma-Aldrich, Brøndby, Denmark. Novozymes A/S, Bagsværd, Denmark supplied the lipase enzyme Novozym®435.

Calculation of reaction rates and equilibrium constants:

The reaction kinetics between glycerol and each of the individual lipid components MAG, DAG and TAG were chosen to experimentally illuminate the forward reaction kinetics of equation 1 to 3 with K_1-K_3 representing the equilibrium constants.

$$\text{TAG} + \text{gly} \xrightarrow[K1]{} \text{DAG} + \text{MAG} \quad (1)$$

$$\text{DAG} + \text{gly} \xrightarrow[K2]{} 2\,\text{MAG} \quad (2)$$

$$2\,\text{MAG} + \text{gly} \xrightarrow[K3]{} \text{DAG} + 2\,\text{gly} \quad (3)$$

Equations (1)-(3) were selected since previous experiments showed that optimal MAG yields were ensured by excess of non-reacted glycerol. Hence, it was found appropriate to include investigations of the reaction behaviour of glycerol with each of the individual lipid components: MAG, DAG and TAG. More equations were refrained from being included to maintain a simple and defined system. Well aware that Eq. 3 is of inconsiderable size compared to 1 and 2 it was included as a 'substitute' for the reverse reaction of Eq. 2 in a 'realistic glycerolysis invironment' with excessive amounts of glycerol being present. From Eq. (1)-(3) initial reaction rates ($-r_a$), equilibrium constant (K_{eq}) and mass balance ratio for the three reactions were calculated as:

$$-r_{TAG} = \frac{d[TAG]}{dt} = [TAG]_t - [TAG]_{t=0\ min} \quad (4)$$

$$-r_{Gly} = \frac{d[Gly]}{dt} = [Gly]_t - [Gly]_{t=0\ min}$$

$$r_{DAG} = \frac{d[DAG]}{dt} = [DAG]_t - [DAG]_{t=0\ min}$$

$$r_{MAG} = \frac{d[MAG]}{dt} = [MAG]_t - [MAG]_{t=0\ min}$$

$$K_1\ (eq) = \frac{[MAG]_{t=eq} \cdot [DAG]_{t=eq}}{[TAG]_{t=eq} \cdot [Gly]_{t=eq}} \quad (5)$$

$$K_2\ (eq) = \frac{[MAG]^2_{t=eq}}{[DAG]_{t=eq} \cdot [Gly]_{t=eq}}$$

$$K_3\ (eq) = \frac{[DAG]_{t=eq} \cdot [Gly]_{t=eq}}{[MAG]^2_{t=eq}}$$

$$Ratio\ (eq) = \frac{[TAG]_{t=0} + [DAG]_{t=0} + [MAG]_{t=0} + [gly]_{t=0}}{[TAG]_{t=eq} + [DAG]_{t=eq} + [MAG]_{t=eq} + [gly]_{t=eq}} \quad (6)$$

Following molecular weight were used for the calculations: GLY: 92.11; FAE: 335.98; FFA:280.5; MAG: 354.09; DAG: 616,07; TAG: 879.55 g/mol.

Enzymatic glycerolysis conducted with pure MAG, DAG and TAG raw material:
Enzymatic glycerolysis were conducted batch wise as reactions between glycerol and either MAG, DAG or TAG in a TP medium. In all reactions, the molar ratio between glycerol and the FFA from the tree different acylglycerols were kept equal at 1.67. A desirable molar ratio between glycerol and TAG of 5, previous found to ensure optimal MAG yields were the underlying basis for this ratio selected (Paper I and II). Reaction blends consisted of 10.6 g glycerol, 20.1-24.4 g acylglycerol (MAG, DAG or TAG), 101-116 ml tert-pentanol and 6-7.3 g enzyme to obtain similar proportions in all experiments of 3.7 wt% enzyme, 77.4 wt% solvent and 18.9 wt% glycerol + acylglycerol. The mixtures were kept stirred in capped 250 ml flasks at a temperature of 50°C. The reaction was initiated by addition of the enzyme and was stopped at a set time by withdrawing 2 ml of the reaction mixture, followed by enzyme removal through a syringe filled with cotton wool. 8 samples were withdrawn in the interval from 5 to 240 minutes. Subsequently, samples were flushed with nitrogen to remove solvent. All samples were stored at -20°C prior to GC-analysis conducted as described in Paper I. Samples used for the calculation of the initial reaction rate were withdrawn after 5 min. For the equilibrium constant calculations samples were withdrawn after 240 min. Results are based on average values from two repeated experiments.

Enzymatic glycerolysis conducted with vegetable oils:
Batch wise glycerolysis reactions at varied glycerol to oil ratios were conducted in two different organic media to calculate reaction rates and equilibrium constants listed in equation (4) and (5). Results are based on averages values from two repeated experiments. In the mixed TB:TP media reaction blends consisted of 0.62-54.75 g glycerol, 5.22-59.36 g TAG rape seed oil, 90 ml TB:TP (80:20 vol%), and 3 g enzyme to obtain similar conditions in all 10 experiments of 2.2 wt% enzyme, 53.1 wt% solvent and 44.7 wt% glycerol + TAG. The mixtures were incubated in water bath heated to 40°C under magnetic stirring. Samples used for the calculation of the initial reaction rate were withdrawn after 30 min. For the equilibrium constant calculations samples were withdrawn after 180 min. n the pure TB medium following conditions were used: Reaction blends consisted of 2.2-5.3 g glycerol, 10 g sunflower oil, 54 ml TB, and 3 g enzyme to obtain 5.0-5.2 wt% enzyme, 69.7-73.5 wt% solvent and 21.2-25.4 wt% glycerol + TAG. The mixtures were incubated in capped 250 ml flasks

under magnetic stirring at 50°C. Samples used for the calculation of the initial reaction rate were withdrawn after 5 min and after 240 min for the equilibrium constant calculations.

Evaluation of the reaction mechanism with pure MAG, DAG and TAG as raw material*:*
In Figure 1 are the time courses obtained from testing the three different acylglycerol materials in combination with glycerol. Fig. 1 illustrates that all three reactions converge towards similar MAG and DAG equilibrium content independent of the starting material. In all set-ups, hardly any TAG (less than 0.2 wt%) was left after reaction and only variations in the MAG, DAG and GLY content, that are believed minor in an overall consideration were attained (Table 2).

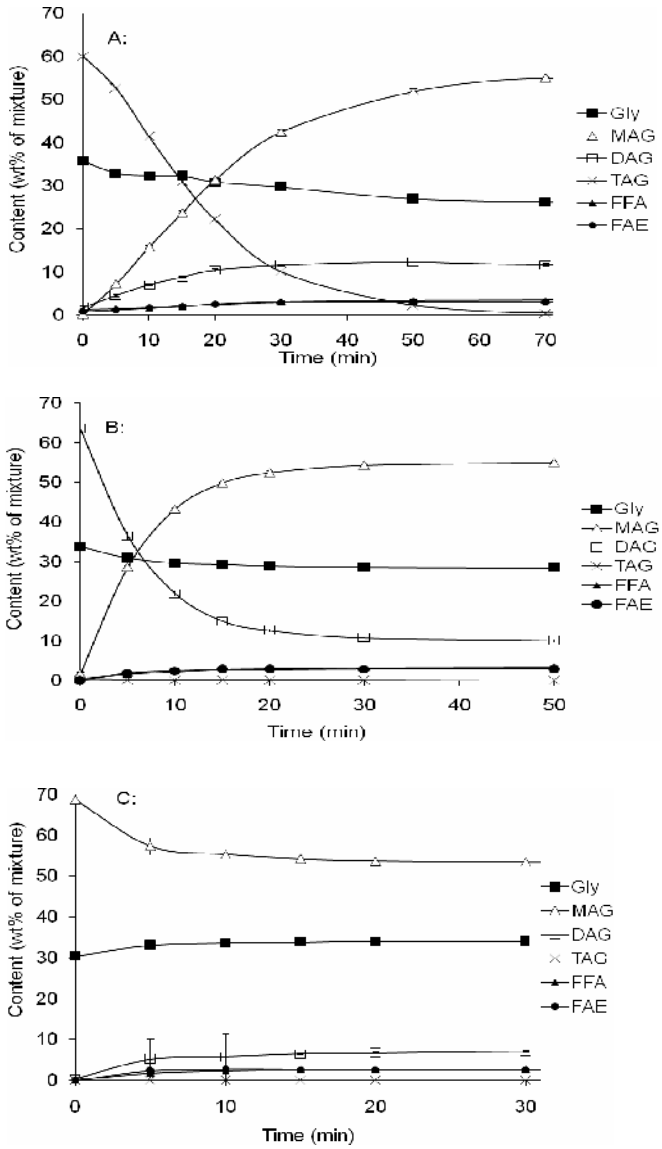


Figure 1: Time courses obtained from enzymatic glycerolysis in a tert-pentanol system conducted with A: TAG, B: DAG and C:MAG as starting material.

Table 2: MAG and DAG content and calculated MAG to DAG ratio after enzymatic glycerolysis for 240 min. in a tert-pentanol system conducted with TAG, DAG and MAG from rapeseed oil.

Reaction medium	Equilibrium content (wt%)			MAG:DAG Ratio (wt%)
	DAG_{eq} ± stdev	MAG_{eq} ± stdev	GLY_{eq} ± stdev	
TAG	11.1 ± 0.2	53.6 ± 0.1	25.8 ± 0.1	84:16
DAG	9.8 ± 0.4	54.0 ± 3.9	28.5 ± 0.7	85:15
MAG	7.31 ± 0.2	53.8 ± 1.5	34.3 ± 0.6	88:12

The MAG, DAG and gly ontent obtained at equilibrium (Table 2) implies that it is not feasible to push the reaction to only MAG formation without reverse reactions spontaneously occurring. When glycerol was present in excessive amounts, the initial amount of MAG or DAG present in the system seemed to have only a minor effect on the equilibrium distribution. In contrast the glycerol concentration appeared to be the predominant factor determining the proportion between MAG and DAG at equilibrium. This points to a correlation between the glycerol and TAG molar ratio for the outcome of the glycerolysis reaction. At the tested glycerol to FFA molar ratio of 1.67 (identical to a glycerol to oil molar ratio of 5) produced MAG appears in abundant amounts compared to the others (5.3 to 7.3 times more MAG than DAG) (Table 2). This verifies that excessive amounts of glycerol are a beneficial choice for the glycerolysis reaction to favor the acylglycerol conversion to MAG rather than DAG. In accordance with that, comprehensive screening experiments confirm that smaller amount of glycerol results in diminished MAG to DAG ratios as illustrated in Table 3.

Table 3: MAG to DAG ratio obtained from enzymatic glycerolysis Performed at varied glycerol to oil molar ratios .

Organic media	Gly:oil molar ratio before reaction	MAG:DAG Ratio (wt%)
TB	2.1	62:38
	2.6	68:32
	4.2	76:24
	5.1	79:21
TB:TP	0.1	7:93
	1.1	40:60
	2.1	61:39
	4.1	75:25
	6.1	80:20

For the MAG system, equilibrium conditions was reached after just 10 minutes (Fig. 1C) compared to 30 min for the DAG system (Fig. 1B) and 70 min. for the TAG system (Fig. 1A). This implies that the sequential reaction with MAG as starting material (Eq. 3) reached a plateau sooner than reactions with DAG which was faster than TAG (Eq. 1 & 2). In the DAG system, the MAG formation appeared synchronous to the DAG degradation (Fig. 1B). In contrast, the TAG system showed a faster DAG formation (equilibrium reached after just 30 minutes) compared to the slower concurrent MAG formation and TAG degradation (equilibrium reached after 70 min.) (Fig. 1A). This suggests a ester-exchange from TAG to MAG, concurrent with the ester-exchange from TAG or DAG to glycerol as illustrated in (Eq. 7):

$$\text{TAG} + \text{MAG} \rightleftarrows 2\ \text{DAG} \qquad (7)$$

This is in agreement with Moquin *et al*. (2006) who observed a faster forward reaction rate for the ester-exchange from TAG to MAG compared to the exchange from TAG or DAG to glycerol (Eq. 1 & 2), making Eq. 7 very plausible/likely. This implies that TAG is converted in the presence of glycerol (Eq. 1) as well as MAG (Eq. 7). Hence, as the MAG amount increases, the forward MAG formation by a sequential ester-exchange between TAG and glycerol (Eq. 1) apparently is slowed down by the competitive exchange between TAG and MAG (Eq. 7).

Evaluation of the initial reaction rate in the MAG, DAG and TAG media:
To look more detailed into the reactions mechanism, calculated initial reaction rates are summarized in Table 4.

Table 4: Calculated initial reaction rates for the main components obtained from enzymatic glycerolysis in a tert-pentanol system conducted with TAG, DAG and MAG from rapeseed oil for 5 min at 50°C.

Initial reaction rate (mol/kg · h)	Reaction medium		
	MAG	DAG	TAG
r_{TAG}	-	-	-0.12
r_{DAG}	0.13	-0.64	0.06
r_{MAG}	-0.52	1.12	0.28
r_{GLY}	0.47	-0.45	-0.43
r_{FFA}	0.10	0.06	0.009
r_{FAE}	0.12	0.08	0.01

In general, the reaction rates varied considerably depending on reaction media used and compound investigated (Table 4). In the MAG medium, the reaction rate of MAG conversion was observed at a rate similar to the glycerol formation while the rate of DAG conversion was slower. This implies undesirable MAG conversion when large amounts of MAGs initially are present. The surprisingly high reaction rates for free fatty acid (FFA) and fatty acid ester (FAE) formation suggest concurrently ester-exchange reactions between other alcohols than glycerol and the acylglycerols (Table 4). In the DAG media, the reaction rate for DAG conversion and MAG formation occurred rapid and exceeded all others calculated reaction rates (Table 4). This demonstrates that the ester-exchange from DAG to glycerol happened very fast in the presence of high DAG amounts. In the TAG medium the order of initial reaction rates for glycerol and the acylglycerols were: $r_{GLY} \geq r_{MAG} \geq r_{TAG} \geq r_{DAG}$ (Table 4). A DAG formation slower than the TAG conversion indicates that the DAG formation by Eq. 1 as well as Eq. 7 is the limiting step of the reaction. The relative slow DAG formation in the TAG media compared to the rapid DAG conversion in the DAG media suggests a shift in the reaction rates as the reaction progresses and more MAG and DAG are formed. This shift in reaction rates is supported by similar observations by Moquin *et al* (2006). The increased amounts of emulsifying MAG/DAG components may be the reason for that. When more glycerol can be emulsified in the oil phase it is likely that the reaction rate is speeded up.

Equilibrium constants in reaction systems with pure MAG, DAG and TAG lipid raw material:
Calculated equilibrium constants (K_1-K_3) for the three forward reactions (Eq. 1-3) shows values independent of the lipid raw materials tested (Table 5).

Table 5: Equilibrium constants and calculated mass balance ratio achieved from enzymatic glycerolysis in a tert-pentanol system conducted with pure TAG, DAG and MAG from rapeseed oil for 240 min at 50 °C.

Equilibrium constant	MAG	DAG	TAG
K_1	-	65.36	65.28
K_2	5.24	4.75	5.01
K_3	0.19	0.21	0.20
Mass balance ratio	0.953	0.948	0.975

As expected, the equilibrium constant for the TAG conversion to DAG and MAG (K_1) exceeded the equilibrium constant for DAG conversion to MAG (K_2) and the reverse reaction (K_3) (Table 5). A K_1-value 13 times higher than the K_2-value clearly illustrates that Eq. 1 is the overall reaction dominating the glycerolysis system, with very high conversion degrees of the TAG. A K_2-value 25 times higher than the K_3-value implies that MAG formation is favored from DAG formation (Table 5). The equilibrium constants were validated by comparable reactions conducted over a broader range of glycerol to oil ratios (2-5) in a TB medium (Table 6).

Table 6: Equilibrium constants after glycerolysis for 240 min at varied glycerol to oil ratios in a TB system. Reaction conditions: 3 g enzyme + 10 g sunflower oil, 2.2-5.3 g glycerol + 54 mL tert-butanol, 50°C.

Molar ratio gly: TAG	Equilibrium constant		
	K_1	K_2	K_3
2.1	31.13	4.54	0.22
2.6	32.15	4.29	0.23
4.2	40.54	4.25	0.24
5.1	38.59	5.07	0.20

Although some variations were observed between the two systems, the pattern was believed similar in an overall consideration (Table 5 & 6) with a K_1-value superior in number to the K_2-value superior to the K_3-value (Table 5 & 6). This implies that the achieved equilibrium constants seem to be valid at lower glycerol to oil ratios than 5 and in another organic media.

Reaction behavior of the enzymatic glycerolysis in a organic TB:TP medium:
Varied glycerol to oil ratios was investigated in a TB:TP mixture to clarify the behavior in a broad substrate range. Figure 2 illustrates the obtained time course from these experiments.

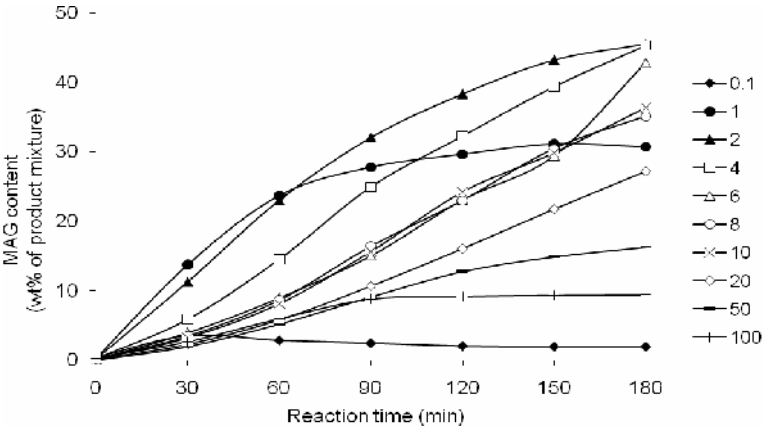


Figure 2: Time courses for MAG contents obtained from enzymatic glycerolysis in a TP:TB system at varied molar ratios of gly to oil.

Table 7 summarizes the calculatd initial reaction rates from the enzymatic glycerolysis performed in the TB:TP media.

Table 7: Calculated initial reaction rate after glycerolysis for 30 min. at varied glycerol to oil ratios in a TB:TP media.

Molar ratio Gly: TAG	Initial reaction rate (mol / kg · h)					
	r_{GLY}	r_{TAG}	r_{FAE}	r_{FFA}	r_{MAG}	r_{DAG}
0.1	$-1.99 \cdot 10^{-2}$	$-4.51 \cdot 10^{-2}$	$4.13 \cdot 10^{-3}$	$2.08 \cdot 10^{-2}$	$2.36 \cdot 10^{-2}$	$4.20 \cdot 10^{-2}$
1	$-6.83 \cdot 10^{-2}$	$-4.74 \cdot 10^{-2}$	$-1.21 \cdot 10^{-2}$	$7.79 \cdot 10^{-3}$	$1.02 \cdot 10^{-1}$	$2.23 \cdot 10^{-2}$
2	$-5.94 \cdot 10^{-2}$	$-4.02 \cdot 10^{-2}$	$2.41 \cdot 10^{-3}$	$3.73 \cdot 10^{-3}$	$8.40 \cdot 10^{-2}$	$1.50 \cdot 10^{-2}$
4	$-5.28 \cdot 10^{-2}$	$-1.63 \cdot 10^{-2}$	$6.77 \cdot 10^{-5}$	$1.66 \cdot 10^{-3}$	$4.32 \cdot 10^{-2}$	$5.56 \cdot 10^{-3}$
6	$1.35 \cdot 10^{-1}$	$-2.28 \cdot 10^{-2}$	$-2.65 \cdot 10^{-3}$	$3.76 \cdot 10^{-5}$	$2.90 \cdot 10^{-2}$	$-2.99 \cdot 10^{-3}$
8	$-2.44 \cdot 10^{-2}$	$-1.63 \cdot 10^{-2}$	$5.18 \cdot 10^{-3}$	$2.48 \cdot 10^{-3}$	$2.46 \cdot 10^{-2}$	$8.79 \cdot 10^{-3}$
10	$-2.57 \cdot 10^{-1}$	$1.90 \cdot 10^{-2}$	$-3.98 \cdot 10^{-3}$	$2.34 \cdot 10^{-3}$	$2.37 \cdot 10^{-2}$	$-1.22 \cdot 10^{-3}$
20	$-7.93 \cdot 10^{-2}$	$6.07 \cdot 10^{-4}$	$-5.04 \cdot 10^{-3}$	$1.07 \cdot 10^{-3}$	$1.65 \cdot 10^{-2}$	$3.75 \cdot 10^{-3}$
50	$-2.68 \cdot 10^{-2}$	$-4.67 \cdot 10^{-3}$	$-2.94 \cdot 10^{-4}$	$2.42 \cdot 10^{-3}$	$1.48 \cdot 10^{-2}$	$1.10 \cdot 10^{-3}$
100	$-3.62 \cdot 10^{-2}$	$-2.45 \cdot 10^{-3}$	$-1.36 \cdot 10^{-3}$	$5.41 \cdot 10^{-4}$	$1.76 \cdot 10^{-2}$	$-7.09 \cdot 10^{-4}$

Overall, the initial reaction rates of the different components varied at the different glycerol to oil ratio but mostly in the same order of magnitude (Table 7). Hence, simultaneous reactions most likely occurred concurrently. At most glycerol to oil ratios (except from gly:TAG ratios of 0.1 and 20), the order of initial reaction rates for the acylglycerols and glycerol were: $r_{MAG} / r_{GLY} \geq r_{TAG} \geq r_{DAG}$ (Table 7) with a MAG formation 3 to 14 times more rapid than DAG formation (Table 7). These findings agree with the findings for the TP system (Table 4). It was not possible to detect a tendency towards an altered reaction rate as the glycerol to oil ratio was increased. Hence, no particular effect of the tested glycerol to oil ratios was observed. Contrary to expectations, extra TAG was formed at glycerol to oil ratios of 10 and 20 and more glycerol was produced than used at a ratio of 6. No obvious explanation was found to that.

Table 8 summarizes equilibrium constants established from the experiments conducted in the TB:TP media.

Table 8: Equilibrium constants and calculated mass balance ratio after glycerolysis for 180 min at varied glycerol to oil ratios in a TB:TP media.

Molar ratio gly: TAG	Equilibrium constant			Mass balance ratio
	K_1	K_2	K_3	
0.1	5.14	1.33	0.75	0.929
1	11.37	3.52	0.28	0.919
2	4.44	3.58	0.28	0.912
4	0.79	2.90	0.35	0.938
6	0.31	1.95	0.51	0.738
8	0.31	1.84	0.54	0.759
10.1	0.44	1.78	0.56	0.989
20.1	-	1.31	0.76	0.917
50.3	-	22.08	0.05	1.024
100.1	-	-	-	-

Surprisingly, it was impossible to support/promote previous calculated equilibrium constants (Table 5 & 6) with the constants obtained in the TB:TP media (Table 8), not even at similar glycerol to oil ratios. Especially the K_1-values were surprisingly low compared to the K_2- and K_3-values. At high glycerol to oil ratios above 4, the excessive amount of non reacted glycerol included in the calculations, definitely diminished the calculated K-values from Eq. 5. This might explain some of the surprisingly low K-values obtained. However, based on the results, it was infeasible to verify that Eq. 1 is the dominating reaction in the glycerolysis system. In accordance with previous findings (Table 5 & 6), the K_2-values in general exceeded the K_3-values (Table 8) although it was to a less extent. This implies that MAG formation is favored from DAG formation with 1.7 to 12.8 times more MAG than DAG formed (glycerol to oil ratios of 50 & 100 not included). From the results it was unknown whether the lower enzyme to substrate ratio influenced the variations from previous findings.

Evaluation of the sn-specificity during enzyme catalyzed glycerolysis:
The time course obtained for MAG and DAG formation in a batch system and the appurtenant distribution among the different sn-isomers are illustrated in Figure 3.

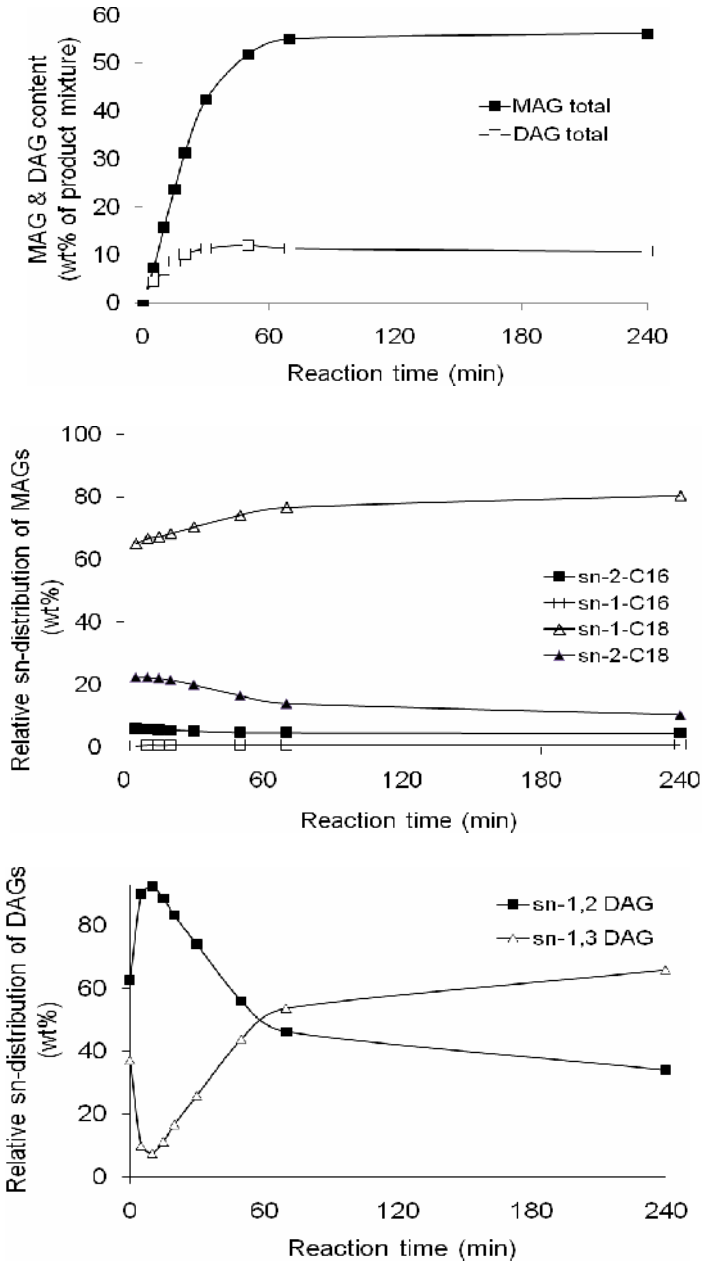


Figure 3: Time course for total MAG and DAG obtained after batch wise enzymatic glycerolysis in a tert-pentanol system and the relative distribution of the different sn-positioned MAG and DAG isomers.

Similar results obtained from glycerolysis in a continuous column reactor are summarized in Fig 4.

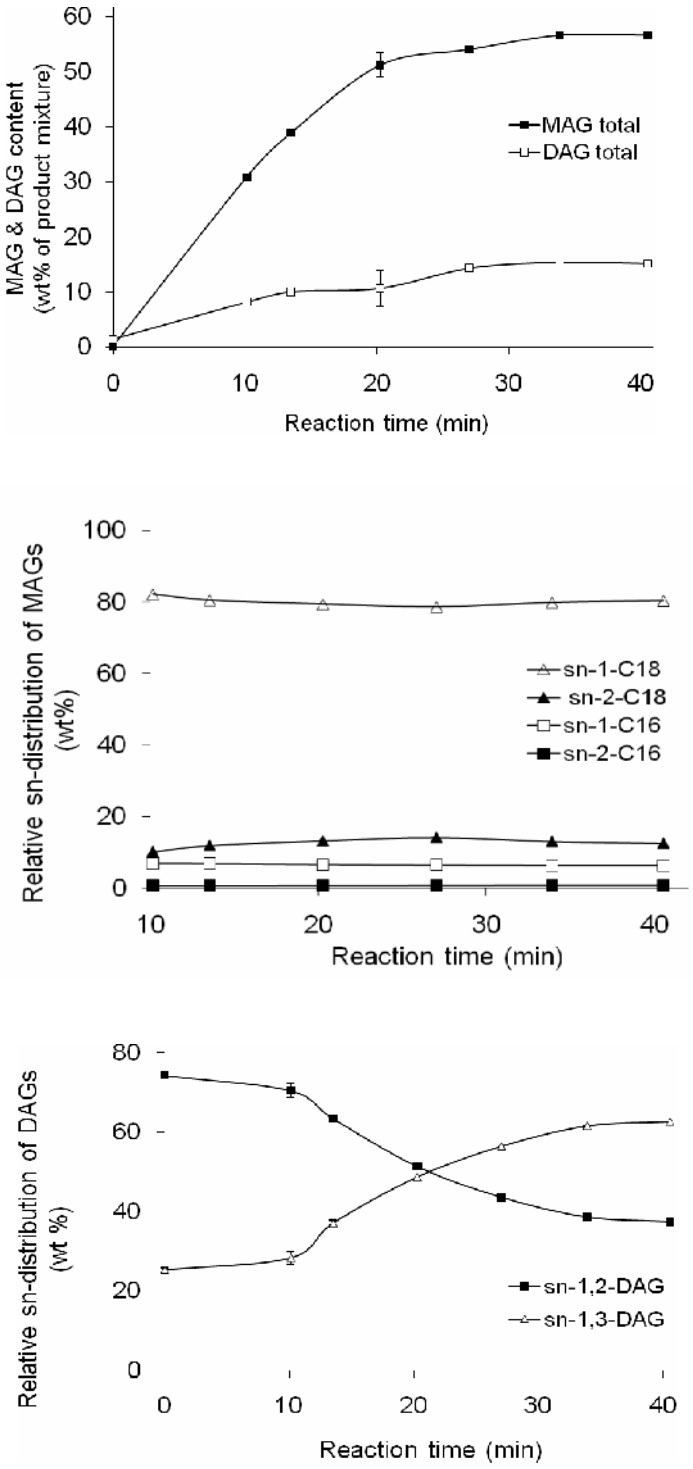


Figure 4: Time course for total MAG and DAG contents obtained after enzymatic glycerolysis in a PBR in a TP system and the relative distribution of the different sn-positioned MAG and DAG isomers.

Overall, similar patterns were observed for the sn-distribution in the batch as well as the PBR reaction system (Fig. 3 & 4). Further details about the relative ratio between the different sn-isomers based on the fatty acid chain length are summarized in Table 9.

Table 9: Relative ratio between the different sn-positioned MAGs and DAGs based on fatty acid chain length (C16 & C18) after enzymatic glycerolysis in a TP medium.

Set up	Time (min)	MAG ratio wt% sn-1:sn-2		DAG ratio wt% sn-1,2:sn-1,3
		C-16	C-18	C-16 + C18
Batch	5	94:6	74:26	90:10
	240	86:14	89:11	34:66
PBR	10	91:9	83:17	70:30
	40	89:11	89:11	37:63

A clear predominance of the sn-1 typed MAGs was observed, accounting for 83 to 94 wt% depending on the reaction system tested and the length of the fatty acid chain (Fig. 3 & 4 and Table 9). In both systems, the C-18 fatty acids, which represented at least 90 wt% of the oil composition (Table 1), showed a tendency to decreased sn-2 MAG ratio as the reaction time was prolonged (Table 9). This suggests an inversion of the MAGs molecules from the sn-2 to the sn-1 position as the reaction progresses. This is believed due to common acyl-migration where the acyl-group spontanuous migrates from sn-2 position to the more stable sn-1 position (Millquist *et al.*, 1996). Hence, conversion of the 2-MAGs to the more stable 1-MAGs obscures the possibility to determine a plausible region-specificity of the lipase when MAGs were formed. Also, in case of enzyme specificity it varies depending on the reaction route from where the MAGs are formed. MAGs obtained from fatty acyl group esterified to a free glycerol molecule indicate acting on the sn-2 position. In contrast, the enzyme indicates preference for the sn-1 and sn-3 position for MAG formed by alcoholysis of TAG or DAG molecules. This provides a very inconclusive picture of the enzymes potential for catalytic effect on specific positions. An observed tendency of the C-16 fatty acid to increase the amounts of sn-2 MAGs as the reaction time was prolonged was totally opposite to the findings for the C-18 fatty acids (Table 9). This hints on plausible enzyme preferences for specific reactions of specific chain lengthened fatty acid residues. This gives an even more complex and unclear picture of the enzyme specificity. Anyhow, the observed distribution among the sn-1 and sn-2 MAGs (Table 9) are close to the commonly equilibrium sn-1:sn-2 MAG ratio of approximately ~ 90:10 obtained from classical glycerolysis (Laszlo *et al*, 2008). Hence, independent of random or specific lipase action, it seemed impossible to affect the final sn-1 to sn-2 ratio from enzymatic glycerolysis, most likely due to acyl-migration.

A shift in the DAG isomers was observed as the reaction time was prolonged with a majority of 1,2-DAG at short reaction times shifting to a majority of 1,3-DAG at longer reaction times (Fig. 3 & 4). A initially majority of sn-1,2 DAGs (Fig 3 & 4) indicates sn-regio specificity of the lipase with preference for acting on the sn-1 (3) position during TAG conversion. However, thermodynamically favorable acyl-migration, as described for MAGs, resulted in a majority of 1,3-DAGs close to the commonly equilibrium sn-1,2:sn-1,3 DAG ratio of approximately ~33:67. Thus, no clear specificity of the enzyme was obtained from the performed investigations.

The sn-distribution after long term usability of the enzyme was compared to previous findings. In Fig. 5 is the different MAG and DAG sn-isomers summed up and Table 10 summarizes the relative ratios.

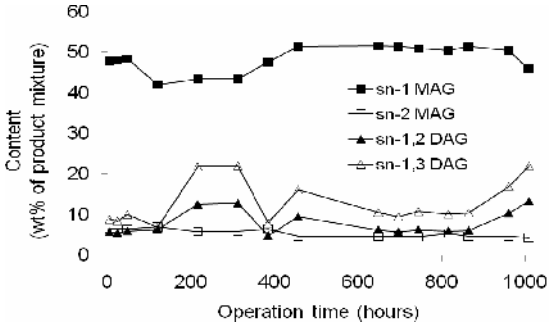


Figure 5: Different MAG and DAG sn- isomers obtained from long term enzymatic glycerolysis in a PBR in a TP system with a reaction time of 30 min in the column.

Table 10: Relative ratio between the different sn-positioned MAGs and DAGs after long term enzymatic glycerolysis in a TP medium in a PBR with a reaction time of 30 min.

Operation time (h)	MAG ratio (w%) sn-1: sn-2	DAG ratio (wt%) sn-1,2: sn-1,3
4.8	88:12	40:60
24	88:12	39:61
48	88:12	37:63
120	86:14	48:52
216	88:12	37:63
312	88:12	37:63
384	88:12	38:62
456	92:8	37:63
648	92:8	37:63
696	92:8	38:62
744	92:8	37:63
816	91:9	37:63
864	92:8	37:63
960	92:8	38:62
1008	92:8	38:62

Long term operations did not alter the different sn-ratios remarkably, indicating that no effect on the enzyme specificity was seen over time (Table 10). All the obtained sn-ratios (Table 10) were close to earlier findings (Table 9) and commonly obtained literature (Kristensen *et al*, 2005; Laszlo *et al*, 2008; Flickinger & Matsou, 2003). Hence, in accordance with expectations, specificity of the enzyme was neither obtained in long term operated enzymatic glycerolysis.

Conclusion

The achieved reaction rates it was difficult to predict a precise glycerolysis reaction mechanism. DAG formation (from TAG degradation) seemed to be the limiting step of the reaction although DAG conversion happened very rapid, when present in high amount. However, it is clear that the glycerolysis system is rather complex with concurrent reactions going and perhaps shifts in reaction rates as the reaction progresses. The investigations of the different DAG and MAG sn-isomers imply some plausible fatty acyl group transfer between the different sn-positions during reaction. However, it was impossible to predict whether these exchanges were caused by acyl-migration or the catalytic effect of the enzyme. Thus, no clear specificity of the enzyme was obtained from the performed investigations. Hence, enzymatic glycerolysis provides MAG and DAG components similar to commonly glycerolysis distributed with equilibrium 1-MAG:2-MAG ratios of ~ 90:10 and 1,2-DAG: 1,3-DAG ratios of ~ 65:35.

Acknowledgement

Bodil Alrø, Danisco A/S is gratefully acknowledged for technical assistance with the GC-FID analysis.

Literature

Flickinger, B. D. ; Matsou, N. **(2003).** Nutritional Characteristics of DAG oil. *Lipids*. 38 (2), 129-132.

Laszlo, J. A.; Compton, D. L.; Vermillion, K. E. **(2008).** Acyl migration kinetics of Vegetable Oil 1,2-Diacylglycerols. *J. Am. Chem. Soc*. 85, 307-312.

Millqvist, F. A.; Vitro, C.; Adlercreutz, P.; & Mattiasson, B. **(1996).** Acyl group migrations in 2-monoolein. *Biocatal. Biotransform*. 14 (2), 89-111.

Kristensen, J. B.; Xu, X.; & Mu, H. **(2005).** Diacylglycerol synthesis by enzymatic glycerolysis: Screening of commercial availble lipases. *J. Am. Oil Chem. Soc*. 82 (5), 329-334.

Moquin, P. H. L.; Temelli, F.; Sovová, H; Saldaña, M. D. A. **(2006).** Kinetic modeling of the glycerolysis-hydrolysis of canola oil in Supercritical Carbon Dioxide Media using equilibrium data. *J. Supercrit. Fluids*. 37, 417-424.

Appendix III

Product Data Sheet Novozym®435

Product Data Sheet

novozymes

Novozym® 435

Valid from 03-Jan-2007

Product Characteristics:

Enzyme Class	Lipase
Declared activity	10000 PLU/g
Colour	Off-white
	Colour can vary from batch to batch. Colour intensity is not an indication of enzyme activity.
Physical form	Immobilized Granulate
Approximate Density (g/ml)	0.40
Carrier	Acrylic resin
Preservatives	Potassium sorbate
	Sodium benzoate
Production organism	Aspergillus niger
	Produced by submerged fermentation of a genetically modified micro organism. The enzyme protein, which in itself is not genetically modified, is separated and purified from the production organism.

Product Specification:

	Lower Limit	Upper Limit	Unit
Propyl Laurate Unit PLU	10000		/g
Loss on Drying 105 C	-	3.0	%

Packaging:

See the standard packaging list for more information.

Recommended Storage:

Best before	When stored as recommended, the product is best used within 6 months from date of delivery.
Storage temperature	0-25°C (32°F-77°F)
Storage Conditions	In unbroken packaging - dry and protected from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperature or higher humidity may lead to a higher dosage requirement.

Safety and handling precautions:

Enzymes are proteins. Inhalation of dust or aerosols may induce sensitization and may cause allergic reactions in sensitized individuals. Some enzymes may irritate the skin, eyes and mucous membranes upon prolonged contact. Powdered enzymes are readily inhaled and should be handled only with specific precautions to prevent inhalation of dust. All equipment and handling procedures must be designed to control airborne dust. Personal respiratory protection is recommended in all cases where full dust control is not secured . All spills, however minor, should be removed immediately. Use respiratory protection. Major spills should be carefully shovelled into plastic-lined containers. Minor spills and the remains of major spills should be removed by vacuum cleaning or flushing with water (avoid splashing). Vacuum cleaners and central vacuum systems should be equipped with HEPA filters. Wear suitable protective clothing, gloves and eye/face protection as prescribed on the warning label. Wash contaminated clothes. A Material Safety Data Sheet is supplied with all products. See the Safety Manual for further information regarding how to handle the product safely.